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REPORT ON

ISOELECTRIC FOCUSING OF PROTEINS AND PEPTIDES

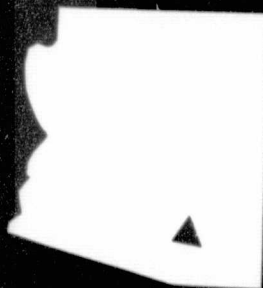
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ISOELECTRIC FOCUSING OF PROTEINS AND PEPTIDES

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I. Operational Parameters of the RIEF Apparatus.

In order to assess the effects of operational parameters (voltage, flow rate, ampholine pH range and concentration, and protein concentration) of the RIEF apparatus on protein resolution, and compare the RIEF apparatus to conventional IEF techniques (flat-bed gel IEF and density gradient stabilized column IEF) with respect to resolution and throughput, it is necessary to obtain a reference protein solution. Requirements for this solution are: (1) It should contain a large number of stable protein components with pI's spanning the 3-10 range, and (2) be amenable to analysis by IEF in that no isoelectric precipitation occurs and that focused bands can be visualized by conventional techniques (staining, UV absorbance). Egg-white protein solution (described below) satisfies these requirements.

Globulin-free (precipitated at half-saturated ammonium sulfate) egg-white proteins were dialyzed against distilled water for several weeks at 4°C. The extended dialysis period was to insure that unstable components would precipitate and result in a solution with a stable protein profile. Analysis of the mixture by IEF in flat-bed polyacrylamide gel (PAG), pH 3.5-10, revealed a minimum of 13 components (visualized with Coomassie Blue/perchloric acid stain), the predominant component was ovalbumin, pI=4.8. The egg-white protein solution is symbolized as ED.

A. Effect of Protein (ED) Concentration on Separation.

Two ED protein concentrations, an order of magnitude difference, were tested: 200 and 2000 mg/liter. Except for the protein concentration the other remaining operational variables were identical for the two runs (applied potential, 200V: flow rate, 10 ml/min

per channel; Ampholine concentration, 0.08% (w/v)). The Ampholine concentration in this and in subsequent runs is considerably lower than that suggested by Ampholine manufacturer for other IEF techniques.

Fig. 1 presents the $A_{280\text{ nm}}$ and pH of each channel for the 200 mg/liter run at the completion of focusing. Fig. 2 presents the PAG (pH 3.5-10) isoelectropherogram of each fraction of the run. Fig. 3 shows the variation of current through the IEF cell during the focusing process. The decrease of current with time, analogous to conventional IEF processes, is representative of all runs and signifies electromigration of ampholytes toward their respective pI's. Figures 4 and 5 are the $A_{280\text{ nm}}$ /pH vs channel and the corresponding PAG isoelectropherogram (pH 3-5) for the 2000 mg run. Results for both experiments are similar in that protein fractionation occurs in three general groups: proteins with pI near 4.8 (ovomucoid and ovalbumin) focused in channels 2-4; those proteins (conalbumins) with pI 6-7 focused near reservoir 5 (see Hb, pI=7.3, for pH reference); and the protein fraction (lysozyme), pI 10-11, focused in channel 10. Some overloading is apparent in the 2 g run in that protein fractions extend over more cells than with 0.2 g ~~run~~.

B. Variation of pH Gradient and Range.

The egg-white protein mixture was subjected to preparative RIEF with various Ampholine ranges. Except for the Ampholine pH range, all experimental conditions were identical within these experiments: 2 g protein (in 1 liter Ampholine solution), 200V applied across the focusing apparatus, 10 ml/min per channel pump speed, and 0.08% (w/v) Ampholine concentration. The Table below correlates the pH range with the corresponding figures depicting the pH and protein ($A_{280\text{ nm}}$) distribution at steady state.

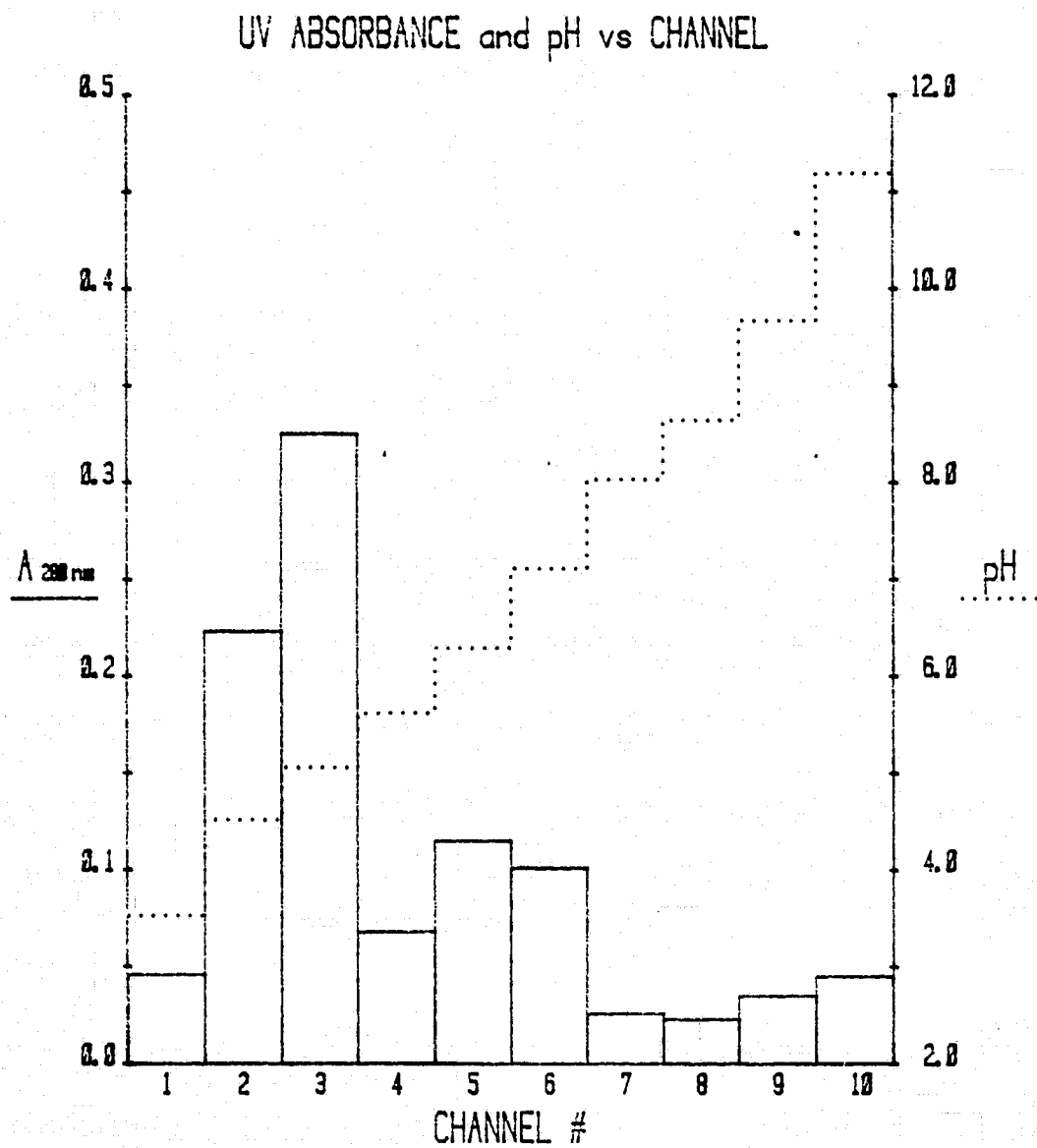


FIGURE 1

uv absorbance and pH vs channel of RIEF apparatus: effect of protein concentration on RIEF, 200mg ED protein/liter

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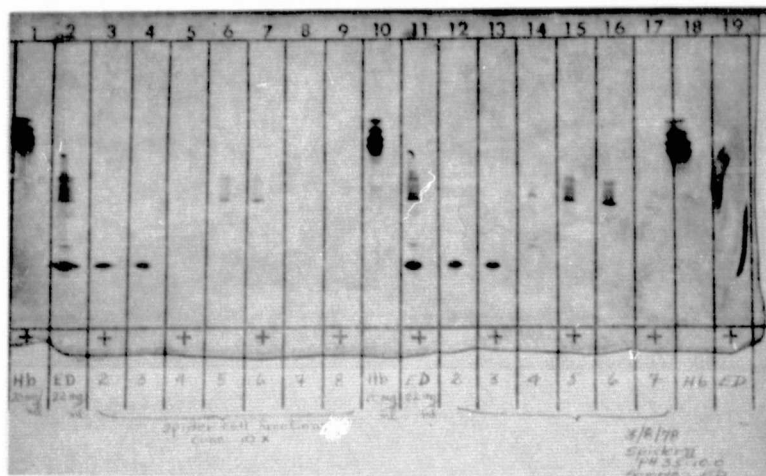
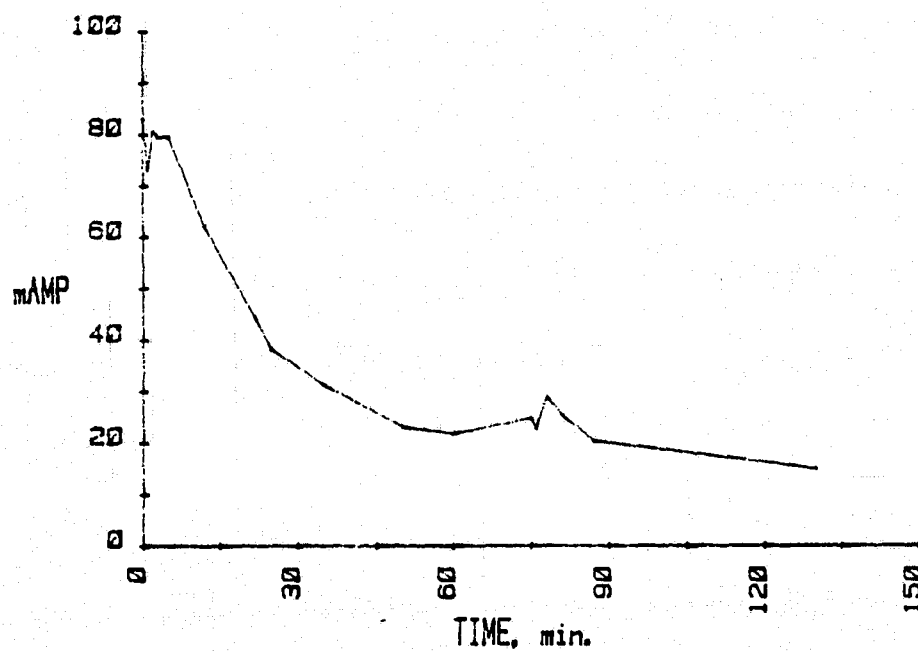


FIGURE 2

PAG isoelectropherogram of RIEF
fractions from 200mg ED protein/liter

FIGURE 3

change in focusing current with time
for RIEF with 200mg ED potein/liter



UV ABSORBANCE and pH vs CHANNEL

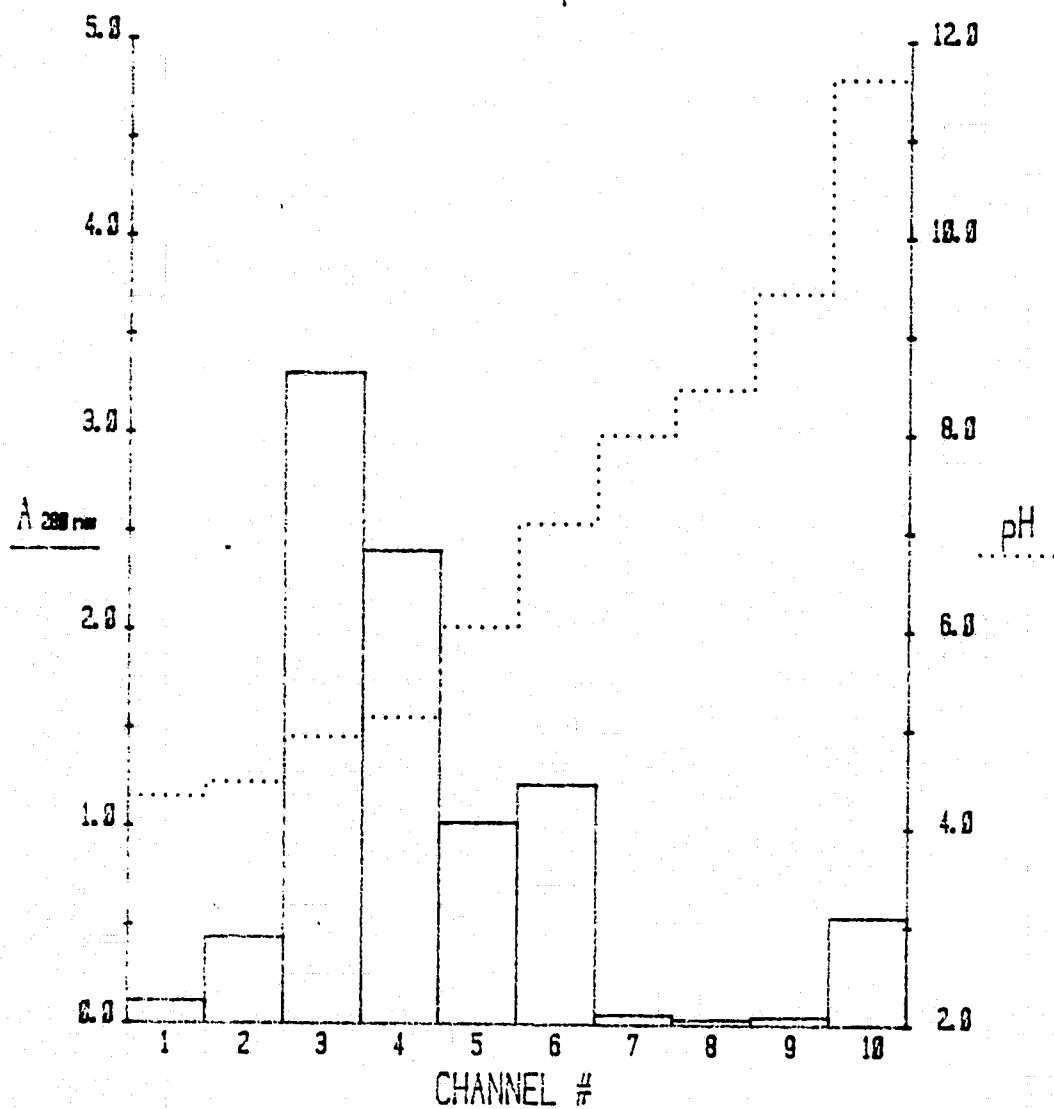


FIGURE 4

uv absorbance and pH vs channel of RIEF apparatus: effect of protein concentration on RIEF, 2000mg ED protein/liter

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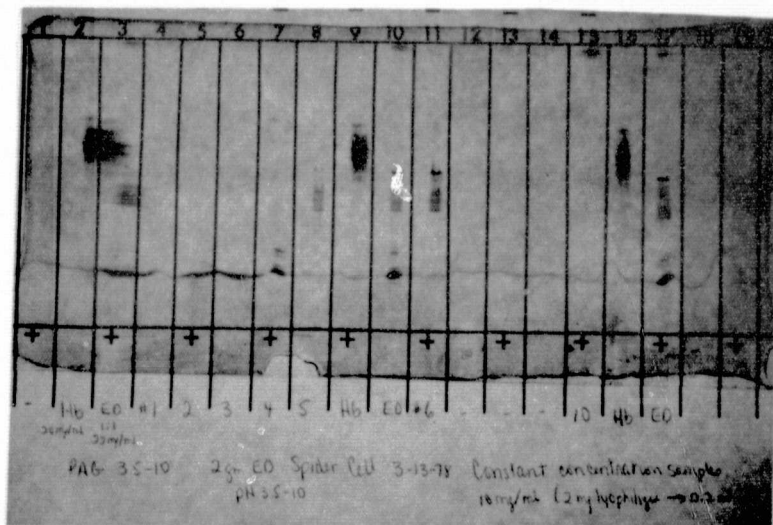


FIGURE 5

PAG isoelectropherogram of RIEF
fractions from 2000mg ED protein/liter

pH range (Ampholine) Fig. #

3.5-10	6
3.5-10	7
3.5-5	8
4-6	9
5-7	10
4.3-5.2	11
no ampholine	12

The relative protein concentrations of any fraction can be approximated as the $A_{280 \text{ nm}}$. The proteins that give rise to these absorbancies are shown by the PAG isoelectropherogram above each channel in the figures. Approximately 18 distinct proteins are observed in the ED reference lanes while no more than 5 proteins, and in some runs only a single protein, are observed in any given fraction. Thus, for these pH regions, enrichment of protein fractions rather than high resolution separations was achieved.

C. Variation of Voltage.

ED was added to prefocused Ampholine, pH 3.5-5 (200V) and the protein mixture was refocused for 75 minutes. The pH and $A_{280 \text{ nm}}$ profiles were noted as well as the protein distribution by PAG IEF. The potential across the cell was then increased to 400V and focusing was continued for an additional 75 minutes. Again the pH and $A_{280 \text{ nm}}$ profile and protein distribution were recorded. The voltage was then restored to the original value and maintained for 75 minutes. Ultra-violet absorbance and pH for each channel 1-10 for the three runs appear in Figures 13-15. In Figure 16 a comparison of the protein distribution by UV absorbance and PAG IEF of corresponding channels for

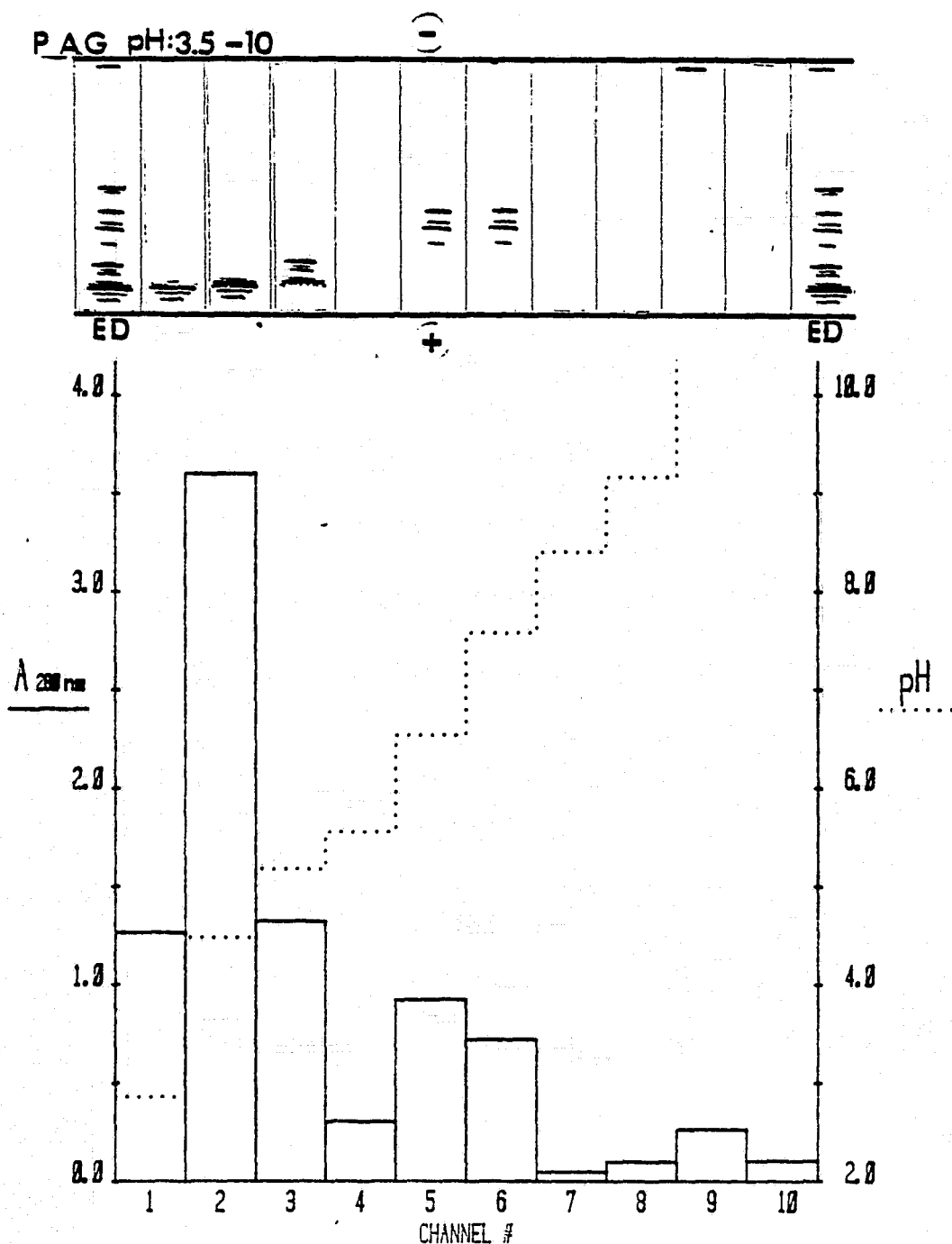


FIGURE 6 uv absorbance and pH vs channel of RIEF apparatus: effect pH gradient and range on RIEF of 2000 μ g ED protein/liter- pH 3.5-10. PAG isoelectropherogram for each channel appears above the respective lanes.

UV ABSORBANCE and pH vs CHANNEL

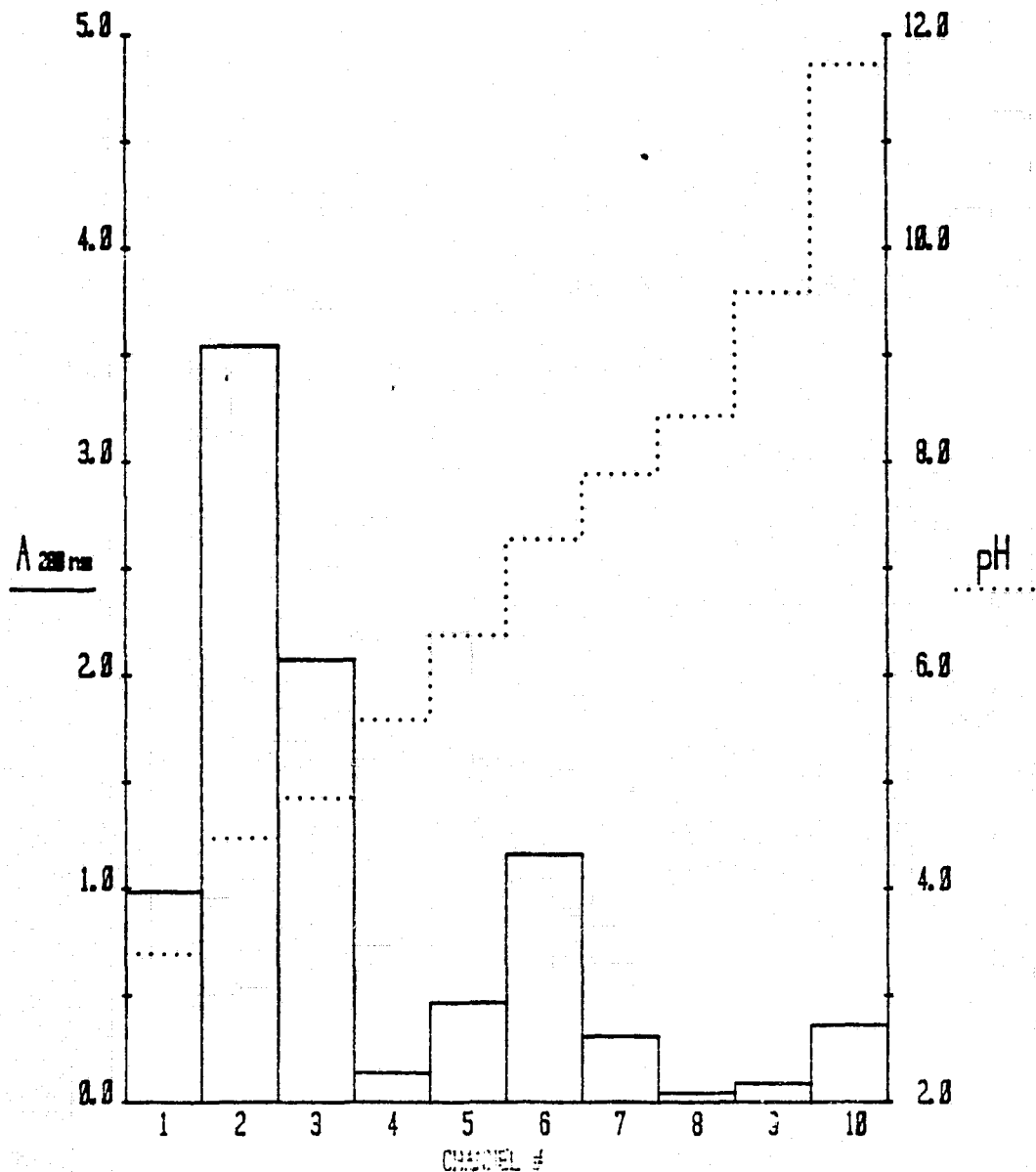


FIGURE 7 uv absorbance and pH vs channel of RIEF apparatus: effect of pH gradient and range on RIEF of 2000mg ED protein/liter- pH3.5-12. PAG isoelectropherogram for each channel appears above the respective lanes.

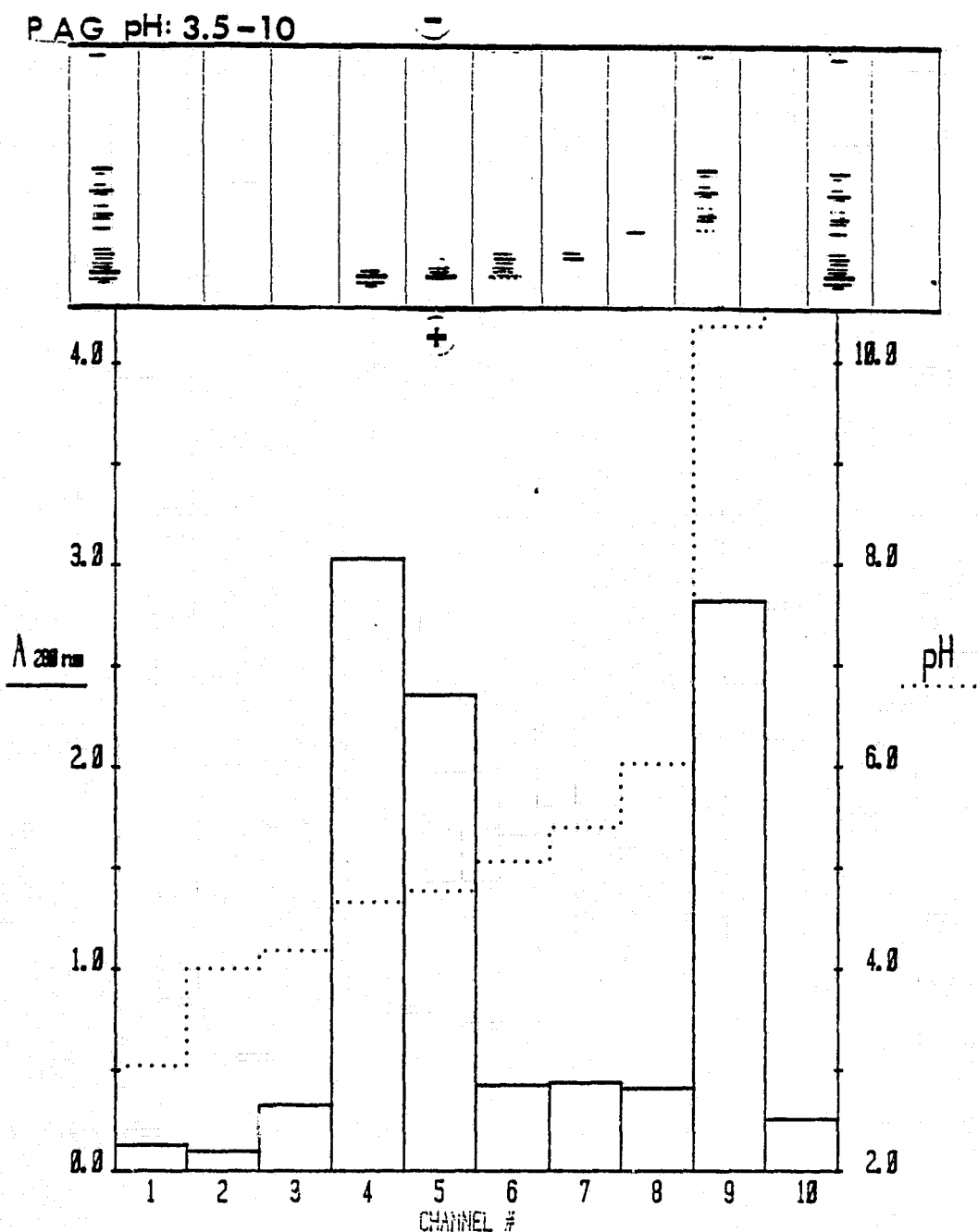


FIGURE 8 uv absorbance and pH vs channel of RIEF apparatus: effect of pH gradient and range on RIEF of 2000mg ED protein/liter pH 3.5-5. PAG isoelectropherogram for each channel. appears above the respective lanes.

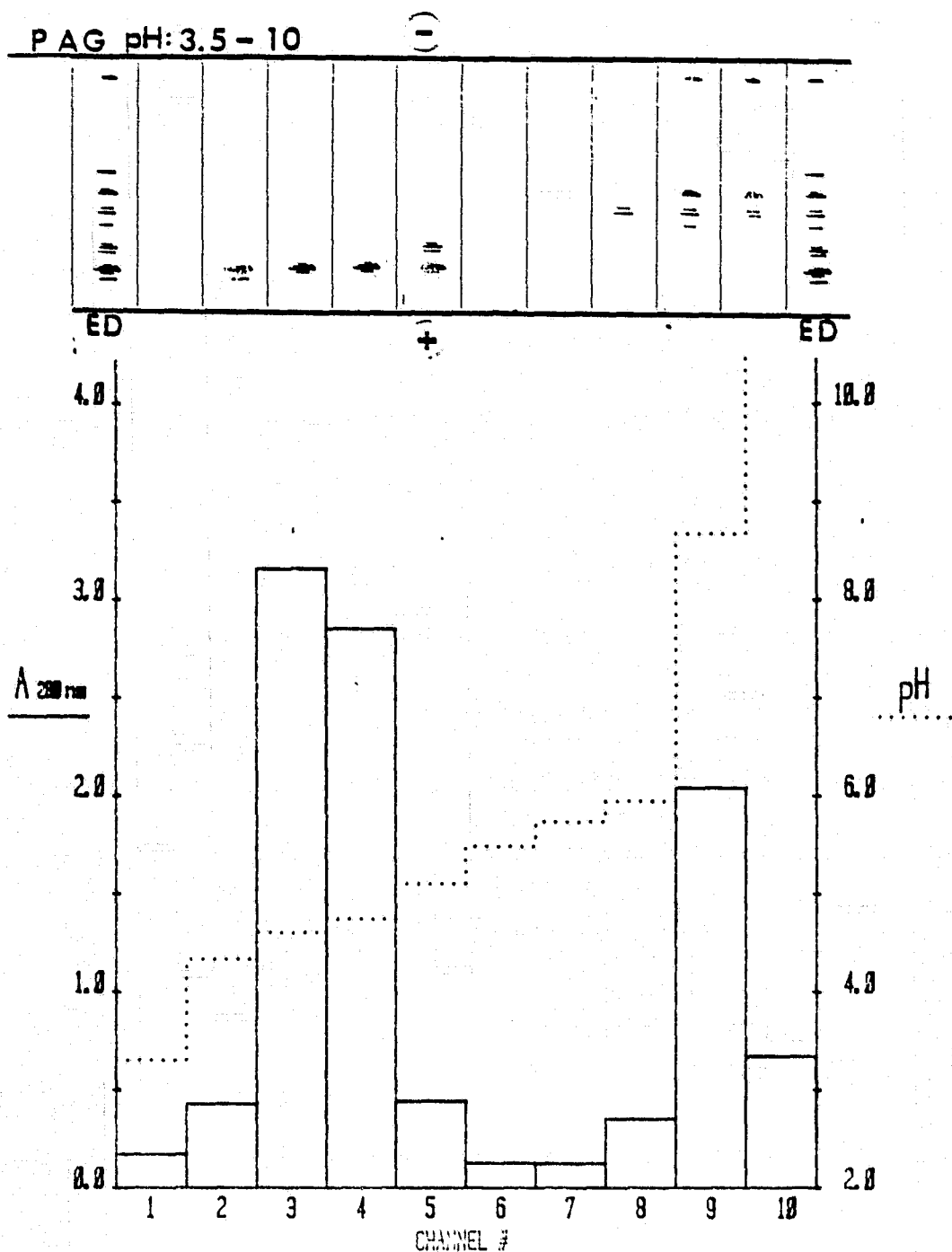


FIGURE 9 uv absorbance and pH vs channel of RIEF apparatus effect of pH gradient and range on RIEF of 2000mg ED protein/liter pH 4-6 . PAG isoelectropherogram for each channel appears above the respective lanes.

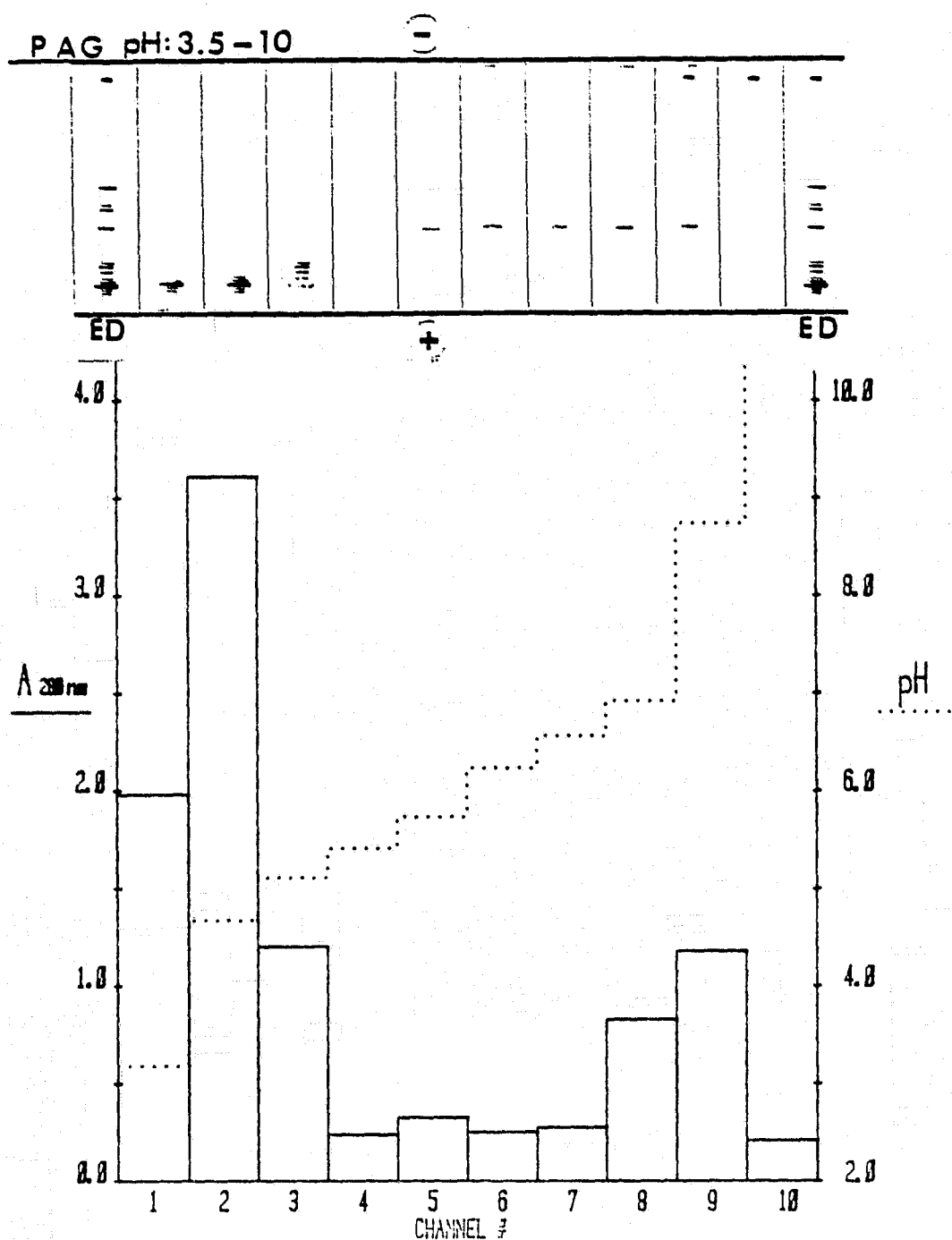


FIGURE 10 uv absorbance and pH vs channel of RIEF apparatus: effect of pH gradient and range on RIEF of 2000mg ED protein/liter- pH 5-7 . PAG isoelectropherogram for each channel appears above the respective lanes.

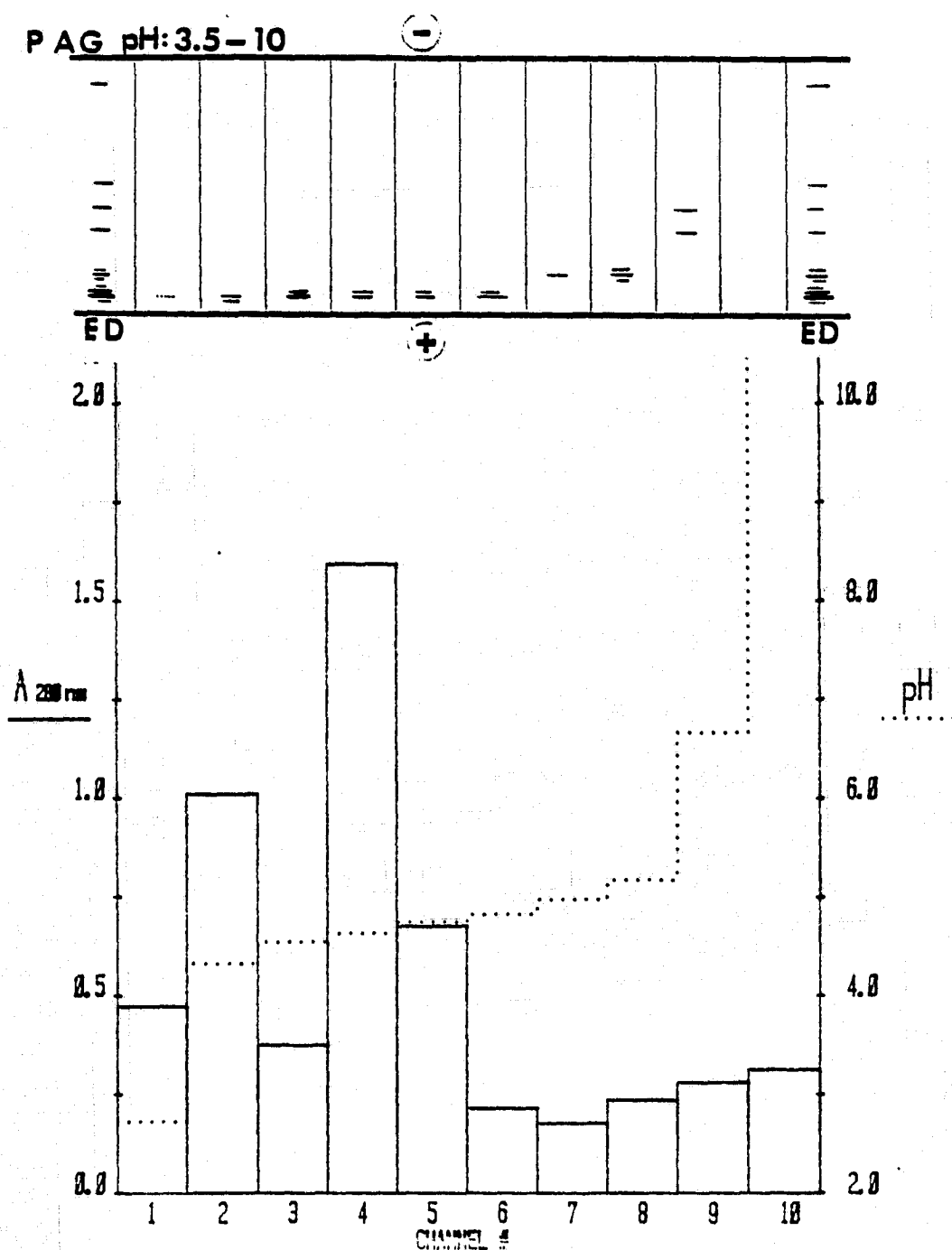


FIGURE 11 uv absorbance and pH vs channel of RIEF apparatus: effect of pH gradient and range on RIEF of 2000 μ g ED protein/liter pH 4.3-5.2. PAG isoelectropherogram for each channel appears above the respective lanes.

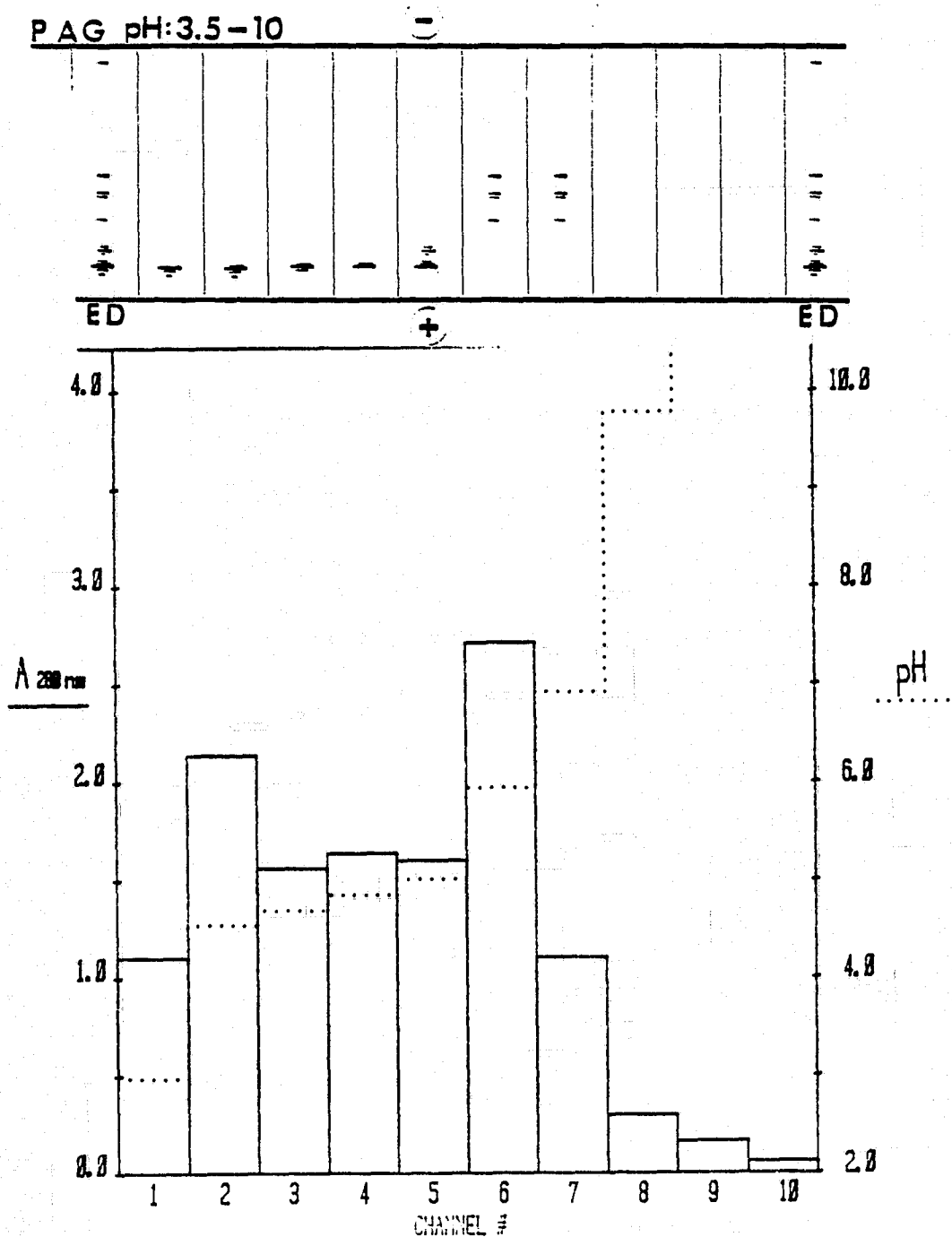


FIGURE 12 uv absorbance and pH vs channel of RIEF apparatus: effect of pH gradient and range on RIEF of 2000mg ED protein/liter- no Ampholine. PAG isoelectropherogram for each channel appears above the respective lanes.

UV ABSORBANCE and pH vs CHANNEL

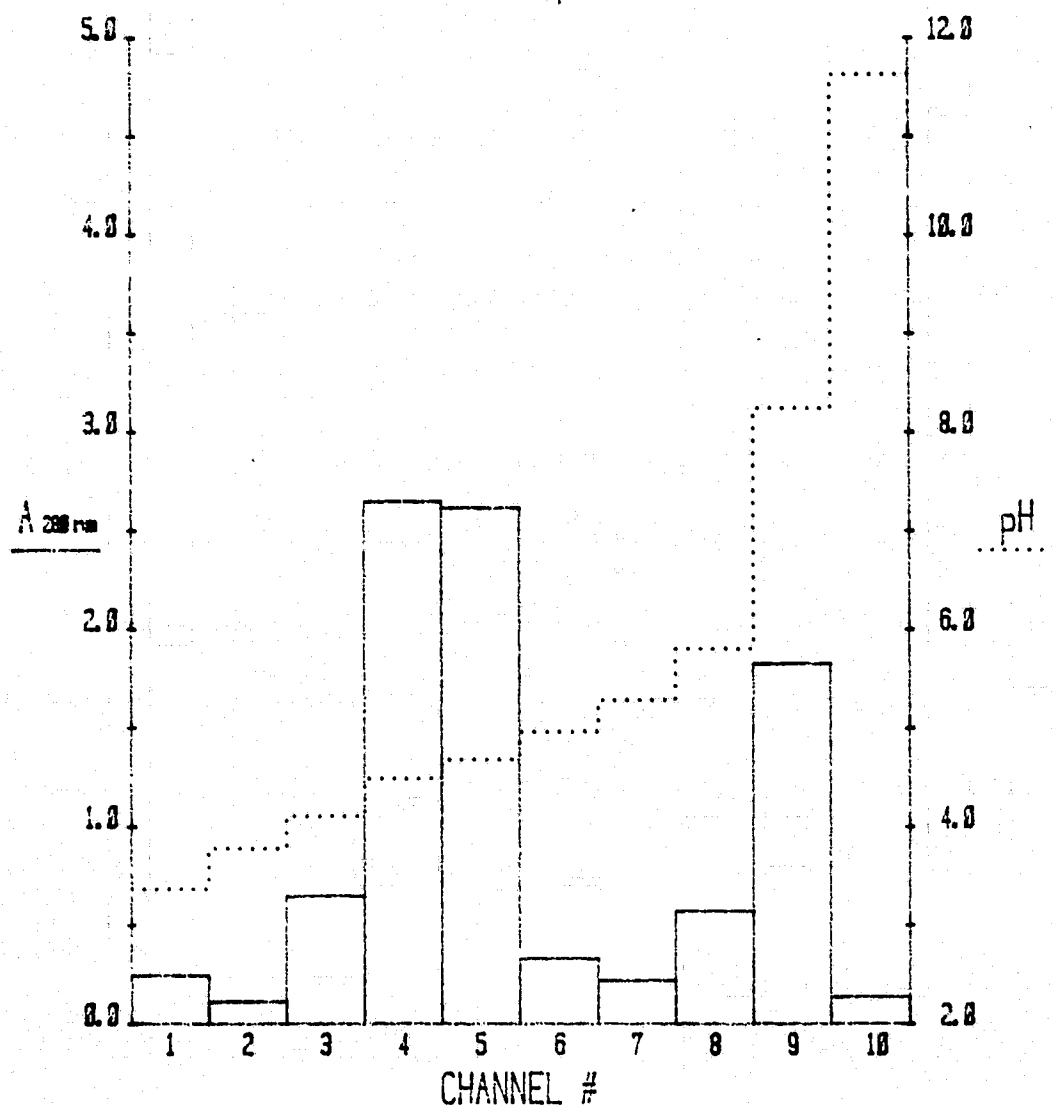


FIGURE 13

uv absorbance and pH vs channel of RIEF apparatus: effect of voltage change on RIEF of ED proteins- results after 75 min. of focusing at 200V.

UV ABSORBANCE and pH vs CHANNEL

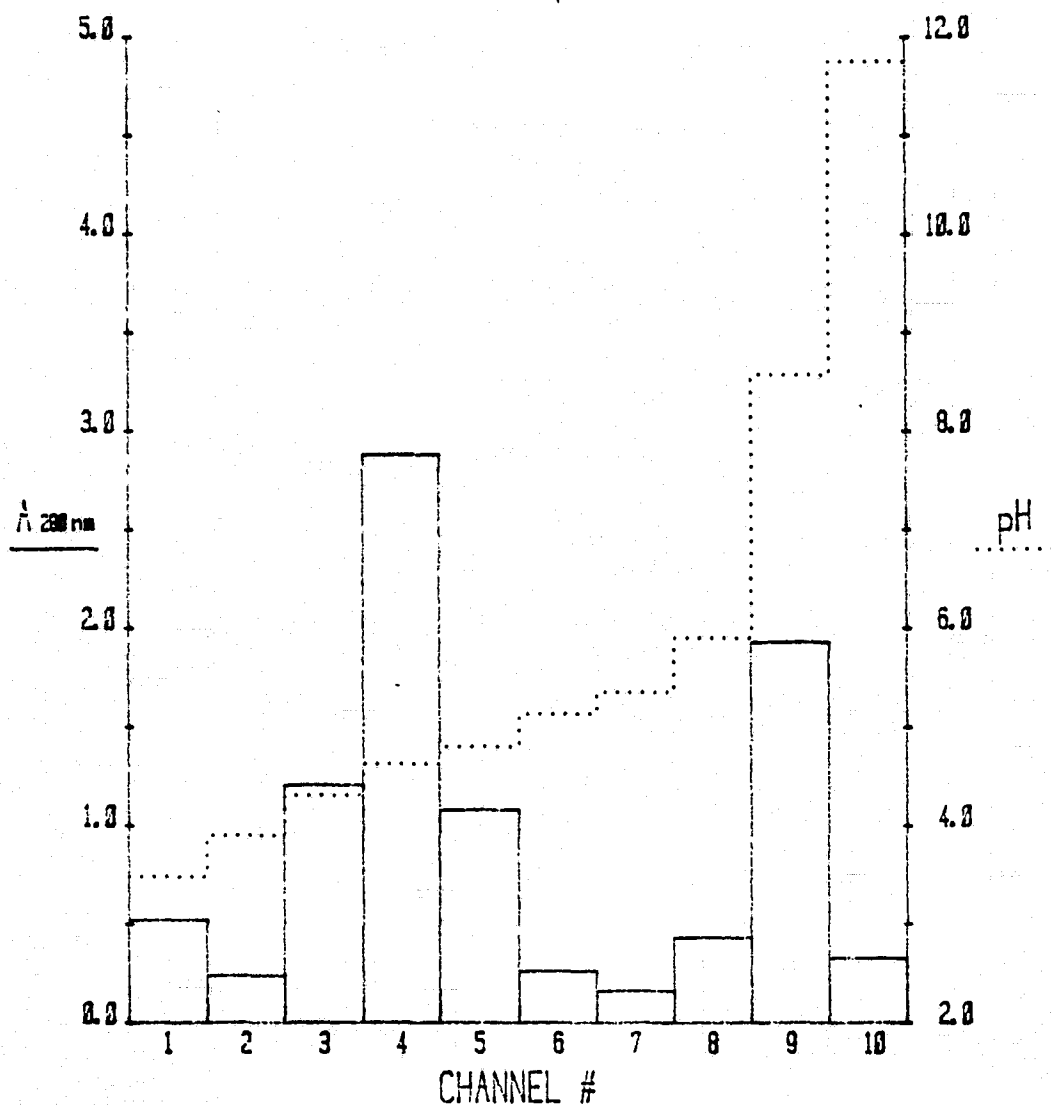


FIGURE 14

uv absorbance and pH vs channel of RIEF apparatus: effect of voltage change on RIEF of ED proteins- results after 75 min. of focusing at 400V

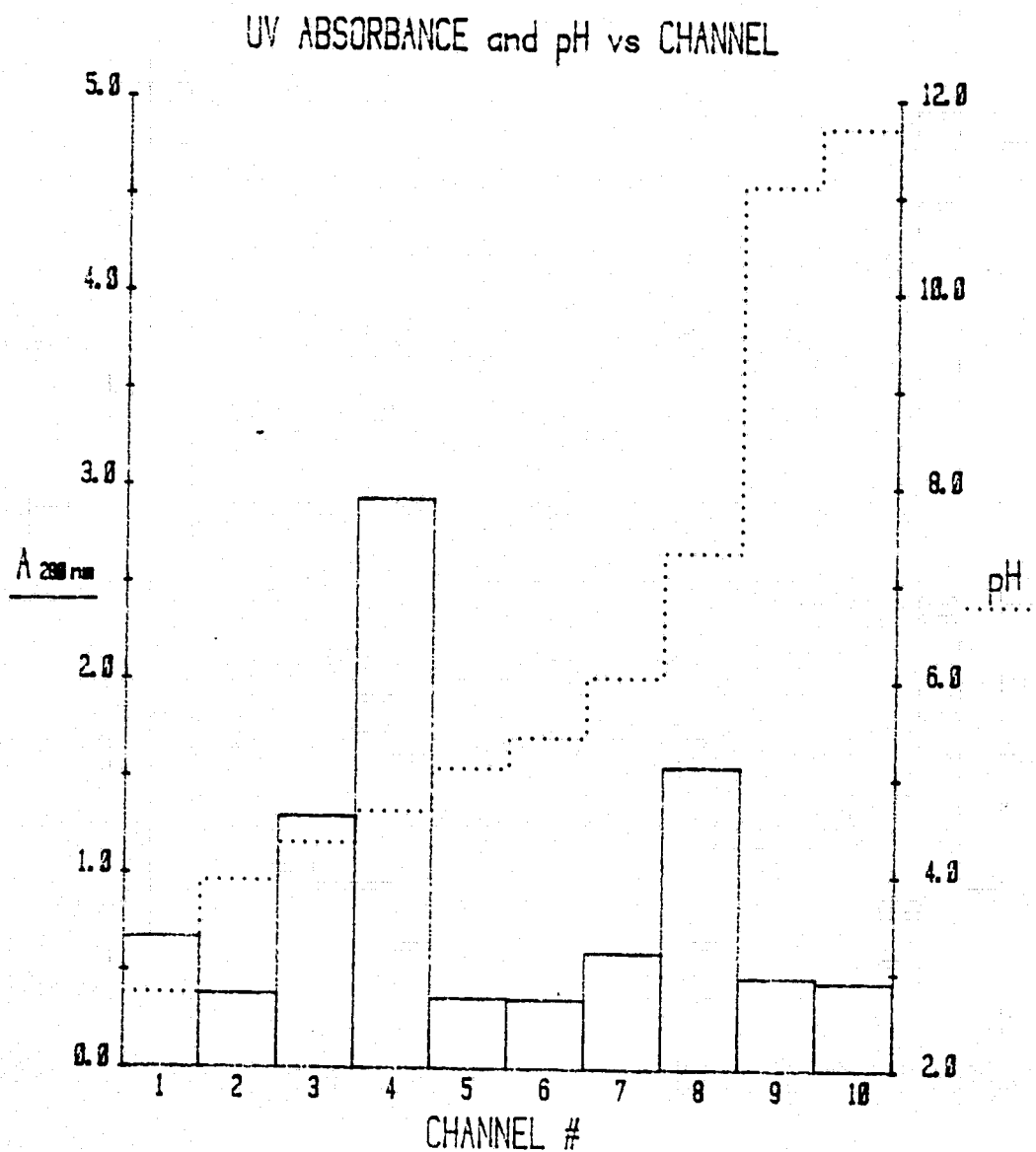


FIGURE 15

uv absorbance and pH vs channel of RIEF apparatus: effect of voltage change on RIEF of ED proteins- results after returning from 400V to 200V.

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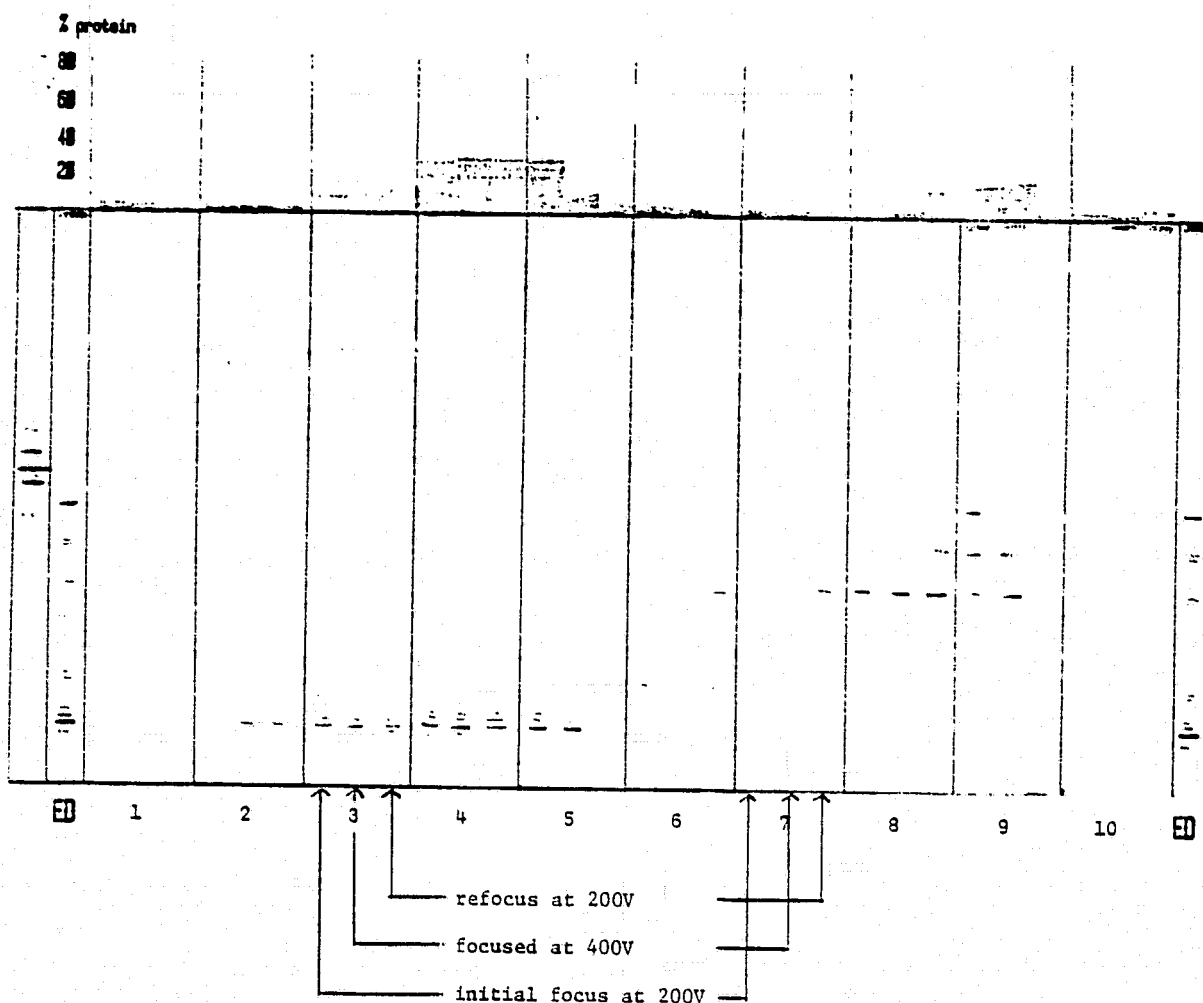


FIGURE 16
effect of voltage on RIEF separation of ED proteins:
isoelectropherogram and % protein of each fraction focused
sequentially at 200, 400, then 200V

the three runs is shown. It can be seen from Figures 13-15 that changing voltage has virtually no effect on the pH gradient and only a minimal effect on protein distribution. The slight variation of protein distribution appears to be a temporal one rather than a function of voltage since restoration to 200V did not return the protein distribution to its original profile.

D. Variation of Pump Speed.

In order to assess the effect of solution flow rate on protein distribution at equilibrium, ED was focused in a pH 3.5-5 gradient as above (10 ml/min per channel, 200V). The protein solution was focused for 75 minutes after equilibrium was attained (estimated from pH and current stability). Analytical samples were collected, the pump speed was reduced 10-fold, and focusing was continued for 75 minutes. Again, analytical samples were collected, the pump speed returned to its original value, and focusing continued for an additional 75 minutes. The results from these experiments appear in Figures 17-19 from which it can be seen that the pH profile is virtually independent of the flow rate. The slight variation in protein profile appears to be temporal and independent of flow rate: the redistribution of proteins observed on changing pump speed from 10 to 1 ml/min per channel was totally absent on changing from 1 to 10 ml/min. Qualitatively, the protein distributions in the corresponding cell compartments are identical for these three conditions as shown by PAG IEF.

II. Comparison of RIEF to Conventional IEF Techniques.

A. Column, Sucrose-Density Gradient Stabilized.

Egg-white proteins (ED) (26 mg) were focused in a sucrose density stabilized solution in a column apparatus (LKB 8100, 110 ml)

UV ABSORBANCE and pH vs CHANNEL

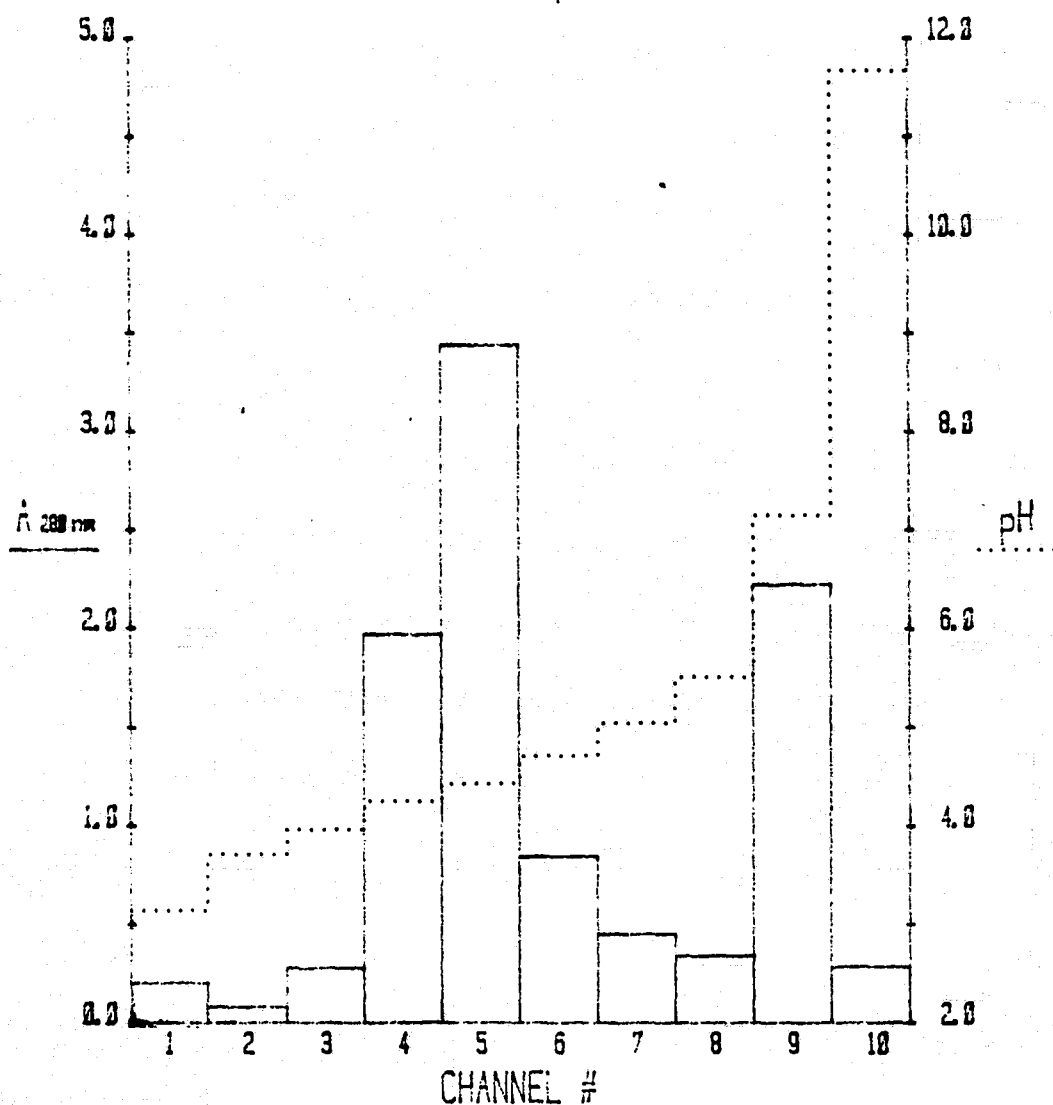


FIGURE 17

uv absorbance and pH vs channel of RIEF apparatus: effect of variation of pump speed on RIEF of ED proteins
pump speed, 10ml/min per channel

UV ABSORBANCE and pH vs CHANNEL

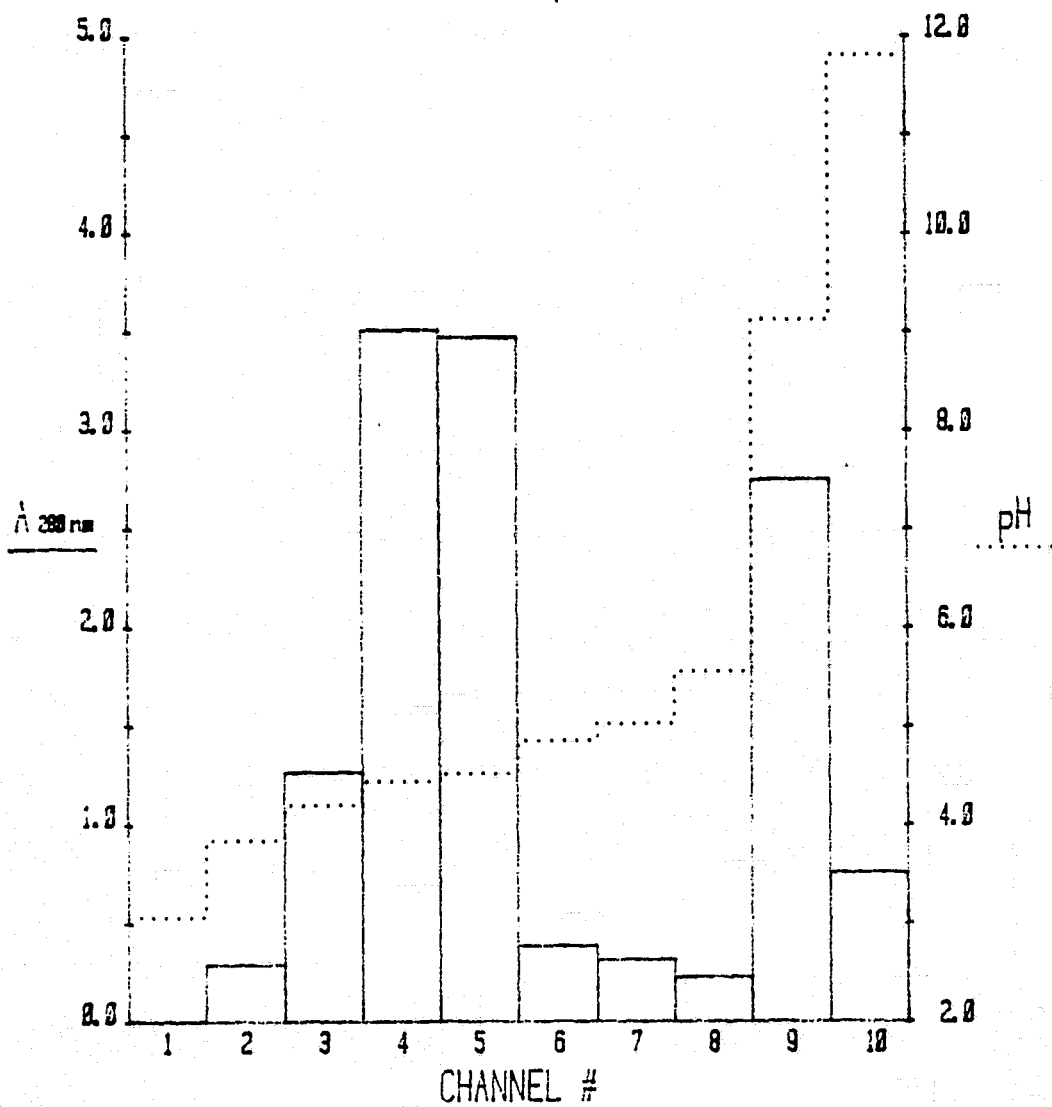


FIGURE 18

uv absorbance and pH vs channel of RIEF apparatus:
effect of variation of pump speed on RIEF of ED proteins
pump speed, 1ml/min per channel

UV ABSORBANCE and pH vs CHANNEL

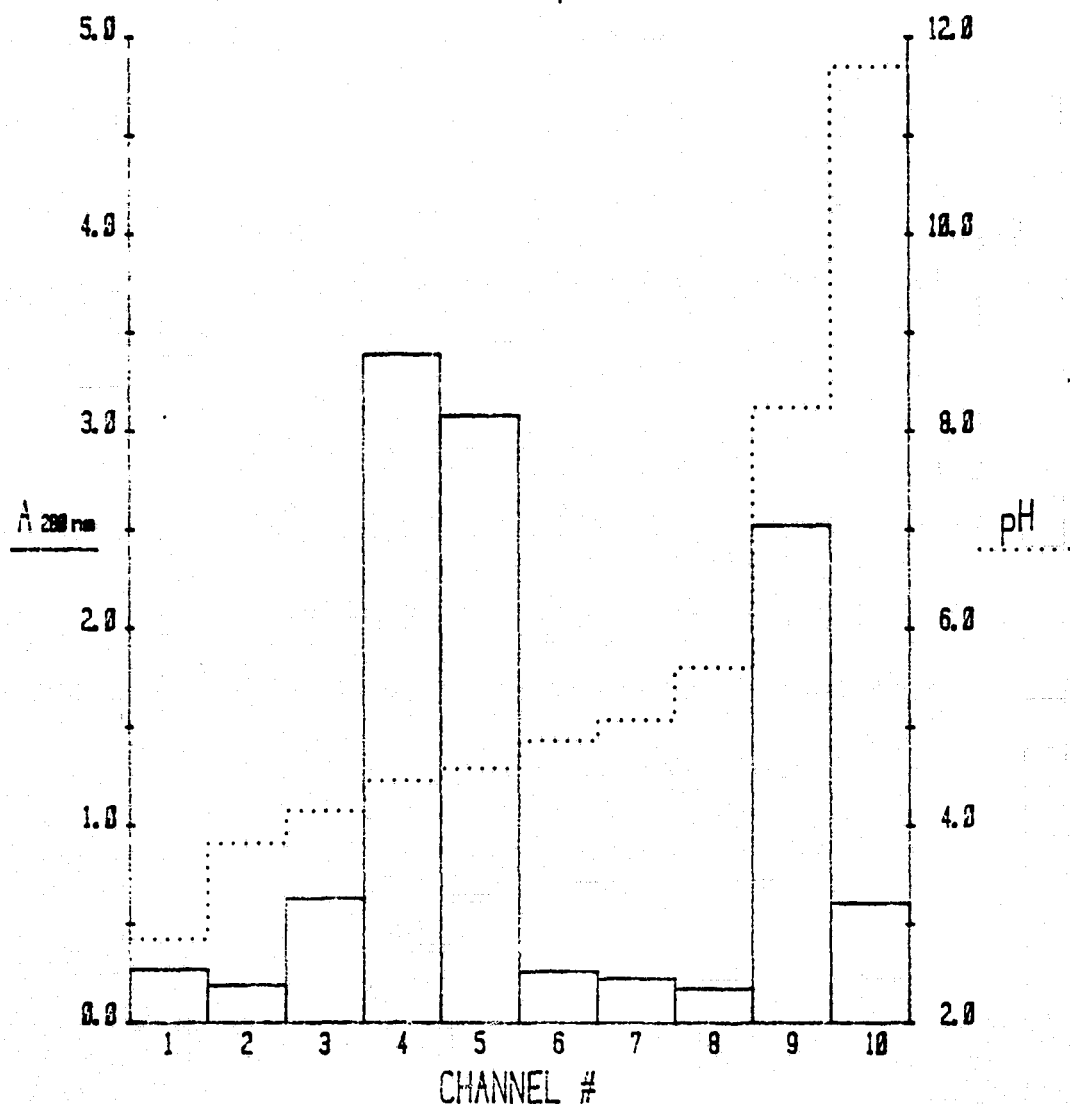


FIGURE 19

uv absorbance and pH vs channel of RIEF apparatus:
 effect of variation of pump speed on RIEF of ED proteins
 pump speed, returning to 10ml/min per channel

to serve as a reference for the RIEF apparatus. After a 24 hr focusing period the solution was eluted through an in-line 280 nm densitometer (LKB) and 3.5 ml fraction were collected (see Figure 20). Fractions were dialyzed against distilled water, freeze dried, redissolved in water, and subjected to PAG IEF analysis.

Comparison of the protein (as $A_{280\text{ nm}}$) vs pH diagram for the column apparatus (Fig. 20) to the corresponding separations with the RIEF apparatus (Fig. 6) reveal some similarities in that the major protein components (ovalbumin, pI 4.8 and conalbumin, pI 6.5) focused with equivalent resolution. Differences between the two methods arise from the quantitative restrictions of the column apparatus (25 mg/zone) and the time requirement (24 hrs). At present for preparative IEF, the LKB column is the apparatus of choice. With the quantity restrictions imposed in the column apparatus, minor components are sufficiently dilute that they are not detected by the LKB in-line photometer. The RIEF apparatus on the other hand processed 2000 mg of protein in just 2 hrs and minor components were collected and detected in their respective compartments.

B. PAG.

PAG IEF offers the highest resolution of the various IEF techniques available. ED was focused in a pH 3.5-10 range flat-bed gel and results of this separation are included in this report to serve as a reference for the resolution of the RIEF device. ED solution (0.2 mg) was applied to a prefocused flat-bed gel and focused for several hours. After focusing a strip of the gel was sliced into 16 0.25 inch segments and each segment was extracted into water for pH measurements. The remaining portion of the gel was fixed

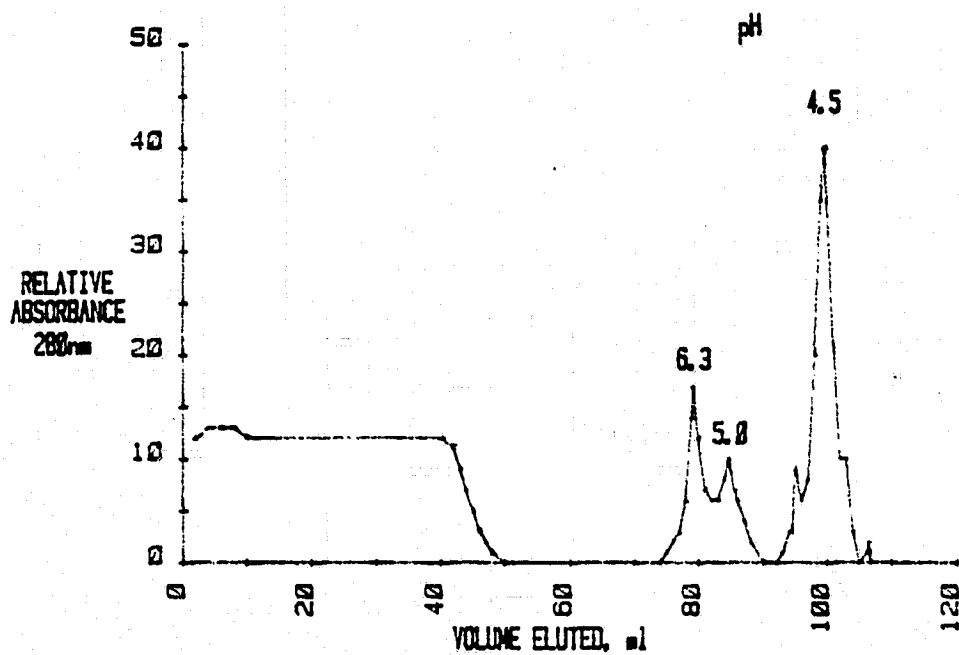


FIGURE 20

uv absorbance vs volume of focused ED protein solution
eluted from LKB 8100 column IEF apparatus

and stained (HClO_4 /Coomassie Blue) and the results of these techniques are reproduced in Figure 21.

To compare IEF in the flat-bed gel to that in the recirculating device, one should imagine the gel axis divided into 10 equal segments, each corresponding to a compartment of the RIEF cell. On comparison of Figure 21 to Figure 6, the protein distribution is roughly equivalent. The resolution in the RIEF apparatus is therefore presumably limited by the number of compartments in the focusing apparatus. On increasing the number of compartments of the RIEF apparatus, the resolution should approach that of flat-bed PAG.

III. Protein and Peptide Separations with the RIEF Apparatus

A. AHF (Antihemophilic Factor, from Abbott Laboratories).

Also called Antihemophilic globulin or clotting Factor VIII, AHF is a globular protein and a normal constituent in human blood, essential for blood coagulation. The third step in the sequence of reactions that comprise stage 1 of coagulation (surface activation of the coagulation scheme to the formation of active thromboplastin) requires AHF. Without AHF the entire sequence of reactions is halted in the first stage and individuals with AHF deficiencies suffer from bleeding disorders (hemophilia). The symptoms of the disorder are dependent upon the levels of circulating AHF, varying from increased bleeding times with skin lesions, to spontaneous hemorrhage. Treatment of AHF deficiencies consists of administration of small volumes of concentrated AHF solutions. The protein is generally obtained from serum as a cryoprecipitate or by precipitation with glycerine.

A sharp pH gradient (pH 3.5-10) was established for 300 ml of 0.08% Ampholine in the RIEF for 2 hrs at 200V. A solution containing

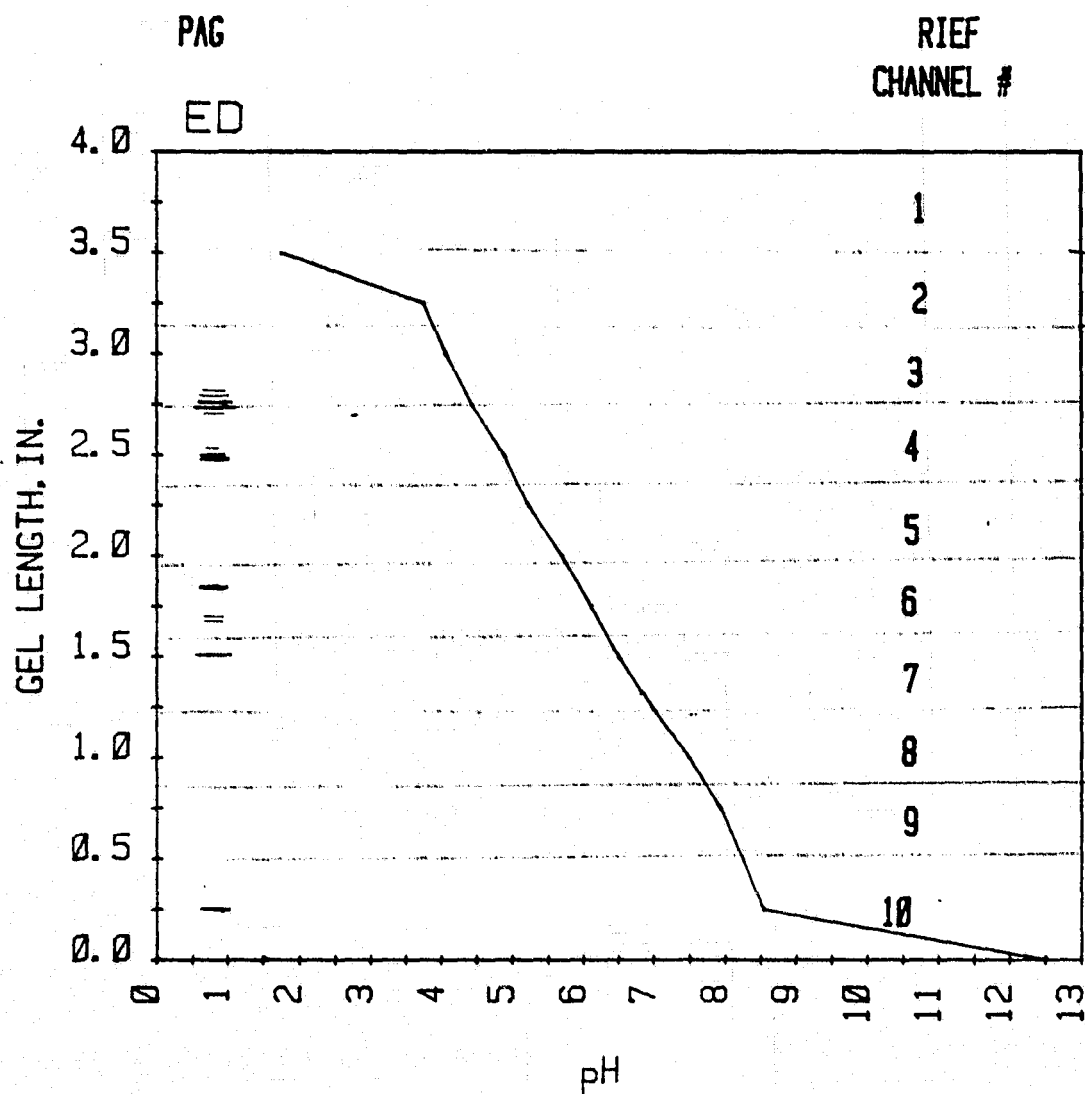


FIGURE 21

PAG isoelectropherogram, pH 3.5-10, of ED proteins:
comparison of resolution by this technique to that
theoretically obtainable with 10-channel RIEF device

300 mg of AHF was added to the pH 7 reservoir and focusing was continued for 15 minutes, equivalent to 5 passes through the focusing apparatus. Fractions were collected and brought to 0.1M citrate for AHF preservation. $A_{280\text{ nm}}$, pH, and clotting activity measurements (APPT) were recorded for the 10 fractions. These results appear in Figure 22. Although a nicely linear pH gradient was established, neither activity (APPT) nor significant protein concentration (as $A_{280\text{ nm}}$) was detected. On careful inspection of the disassembled focusing apparatus, precipitated protein was observed on the filter elements. In an attempt to minimize precipitation the run was repeated with protein concentrations reduced to the minimum required for activity measurements; however, similar results were obtained. IEF of AHF was performed in both flat-bed gels and in a column to determine whether the precipitate was a result of RIEF. Precipitation occurred in both experiments immediately following sample addition. The similarities between the three techniques are (1) the sample solution becomes desalted with the ionic strength of the medium maintained only by the ionic contribution of the carrier ampholytes, and (2) all methods require removal of the joule heat generated by the focusing current. In the case of AHF, precipitation occurs at reduced temperature indicating that the separation must be performed at room temperature or above. However, precipitation caused by reduced ionic strength cannot be ruled out. In this case, possible solutions to the problem are increasing the Ampholine concentration; or adding amino acids, peptides, sugars, or urea to maintain protein solubility; or continuous infusion of dilute salt solution to anode and cathode compartments to maintain a steady state ionic strength. Follow-up experiments are planned.

UV ABSORBANCE and pH vs CHANNEL

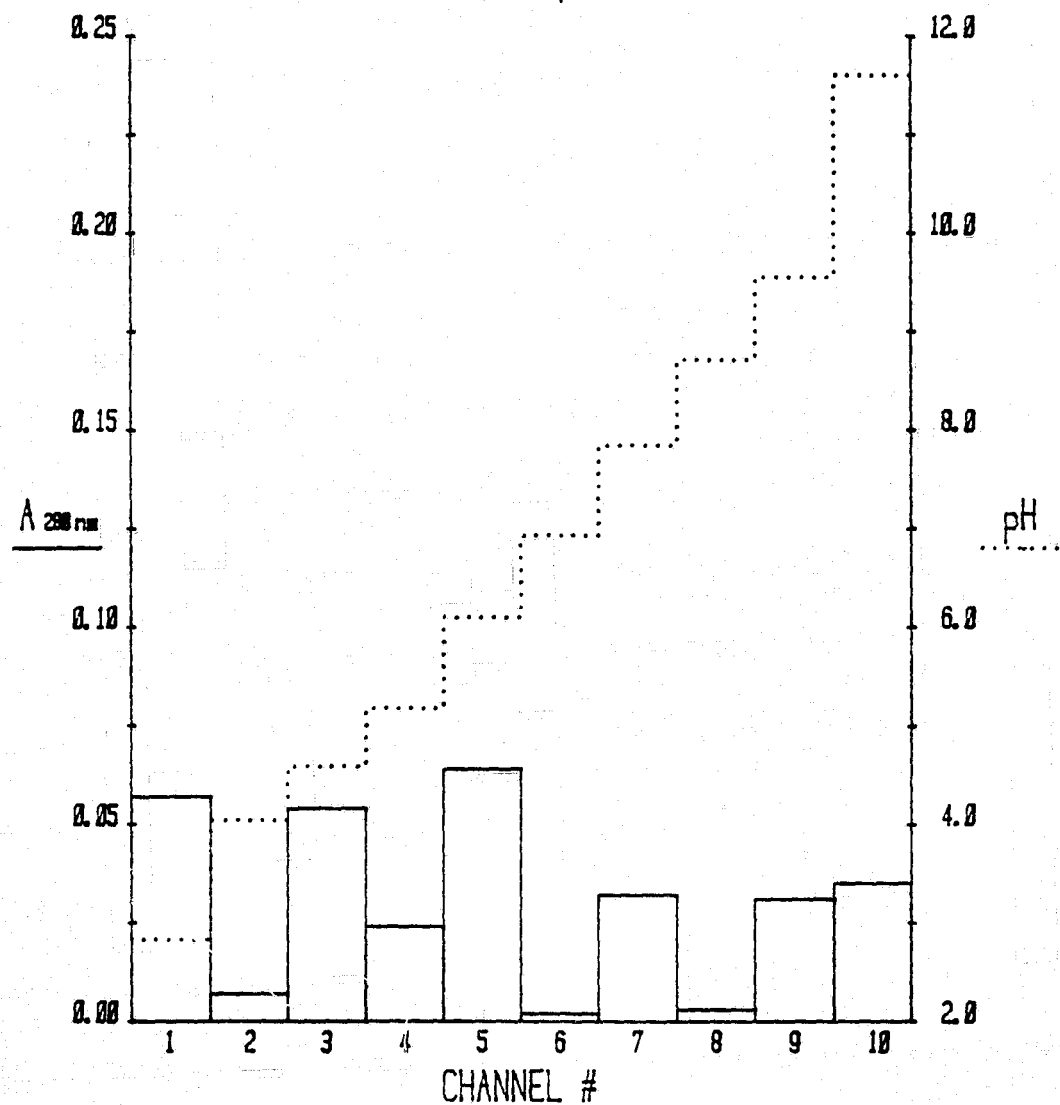


FIGURE 22

uv absorbance and pH vs channel of RIEF apparatus:
results from RIEF fractionation of AHF

B. Thymosin, Fraction 5.

Thymosin, Fraction 5 is a partially purified, partially characterized extract of calf thymus and is a mixture of a large number of polypeptides. A detailed description of the preparation and chemical and biological properties is presented by Low and Goldstein. Briefly, calf thymus is homogenized, extracted with N-saline and octanol and centrifuged. The supernatant is heat treated (80°C), then filtered to remove denatured precipitated proteins. The desired peptides in the resulting filtrate are precipitated with acetone, then collected by filtration. The precipitate is redissolved in phosphate buffer, reprecipitated with ammonium sulfate, collected by centrifugation, then redissolved in Tris buffer and collected as an ultrafiltrate (Amicon DC-2, hollow fiber, 15,000 daltons). Fraction 5 is obtained in solution by desalting the filtrate with Sephadex G-25. Lyophilization yields a white powder.

Fraction 5 is a large group of polypeptides with molecular weights of 1,000-15,000 g/mole. Analysis by PAG IEF (Ampholine, pH 3.5-10) reveals the pI's for the constituents of Fraction 5 span the entire pH range. (α -peptides have pI's below 5, β -peptides in the range 5-7, and γ -peptides above 7). At present, eight components in the α region (α_1 - α_8) and four components in the β region (β_1 - β_4) have been isolated.

Fraction 5 has been successfully administered to athymic or immunodeficient experimental animals and humans to mimic some functions of the intact thymus gland. Increased T cell differentiation and production of antibody-forming cells result from thymus Fraction 5. Based on these observations, thymic peptides have been used for treatment of infectious diseases and in patients with reduced immunological functions,

and as an anti-cancer agent. In addition, it has been suggested that thymosin preparations be used in the treatment of diseases related to the aged since aging, decreased cellular immunity, increased rate of infection, and decreased blood thymosin concentrations and activity are all coincident. Research with thymosin peptides is currently an active area. Dr. Alan Goldstein is the principal contributor. We are presently collaborating with Dr. Goldstein: our goal is to supply his lab with purified thymosin components for biological testing. This material was focused by both the column technique (25 mg) and by the recirculating apparatus (50 mg). Fraction from both techniques were analyzed by PAG IEF, pH 3.5-10, and the results appear in Figure 23. Separation of components was effected by the RIEF apparatus, however, the resolution is significantly lower than with the column. One factor affecting the resolution of any isoelectric focusing technique is the ampholyte buffer capacity. In this light it is important to note that the Ampholine/protein ratio for the column is 25 times of the RIEF experiment. Follow-up experiments are planned.

C. Vasoactive Peptide.

A protein solution containing predominately albumin and a vasoactive peptide as a trace component was supplied by Abbott Laboratories. Isolation of the active peptide was the major interest of Abbott.

A four gram sample dissolved in 37 ml H_2O was added to the center portion of a prefocused pH 3.5-6 gradient (1200 ml x 0.08%w/v Ampholine) and focusing was continued over night. Figure 24 presents the protein and pH distributions. The predominant component is albumin (pI, 4.8), focused in chamber 5, and presumably due to overloading, albumin is also present in #6. Fractions were freeze-dried and sent to Abbott

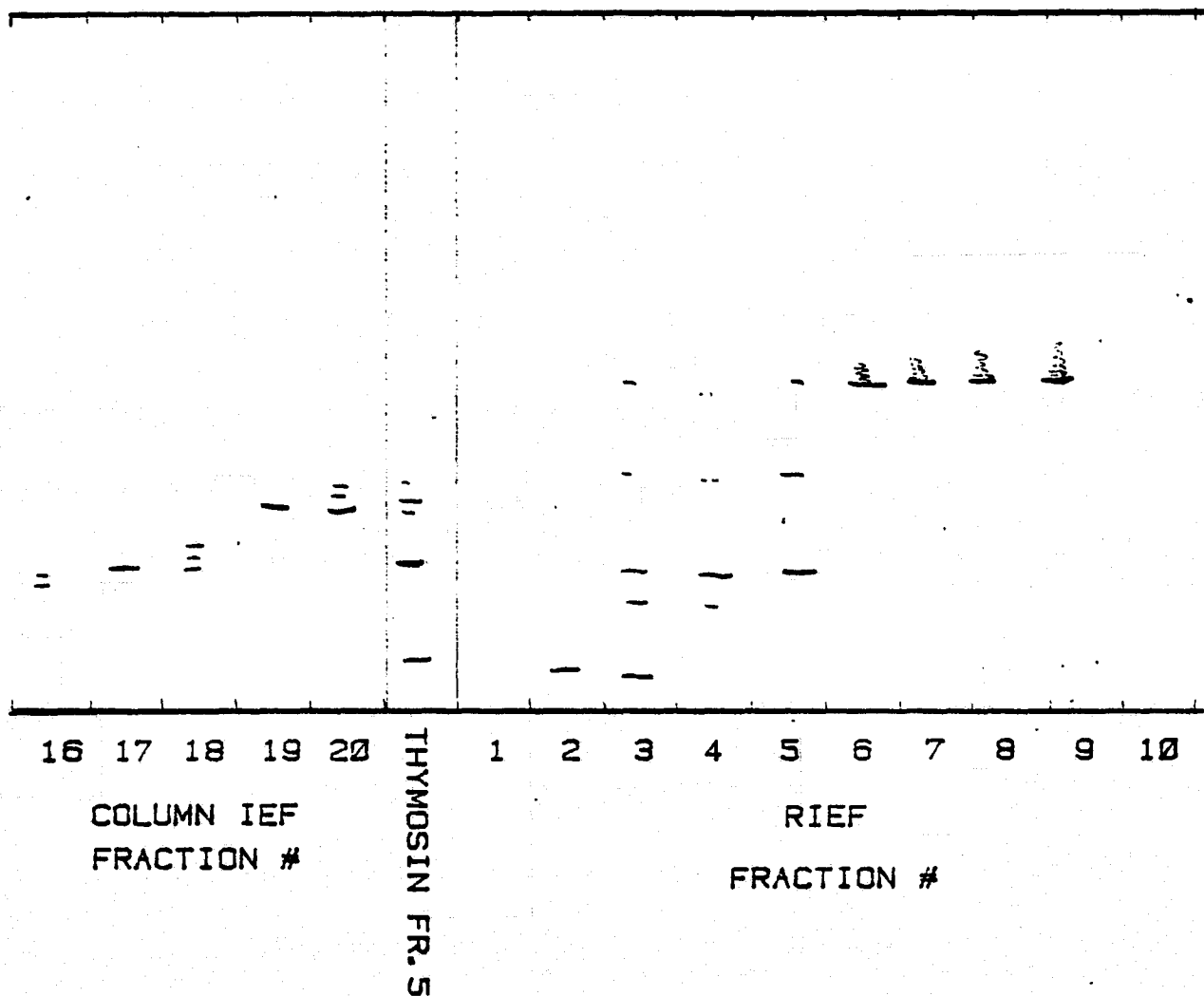


FIGURE 23

PAG isoelectropherogram of thymosin peptides following fractionation by column IEF and RIEF techniques

UV ABSORBANCE and pH vs CHANNEL

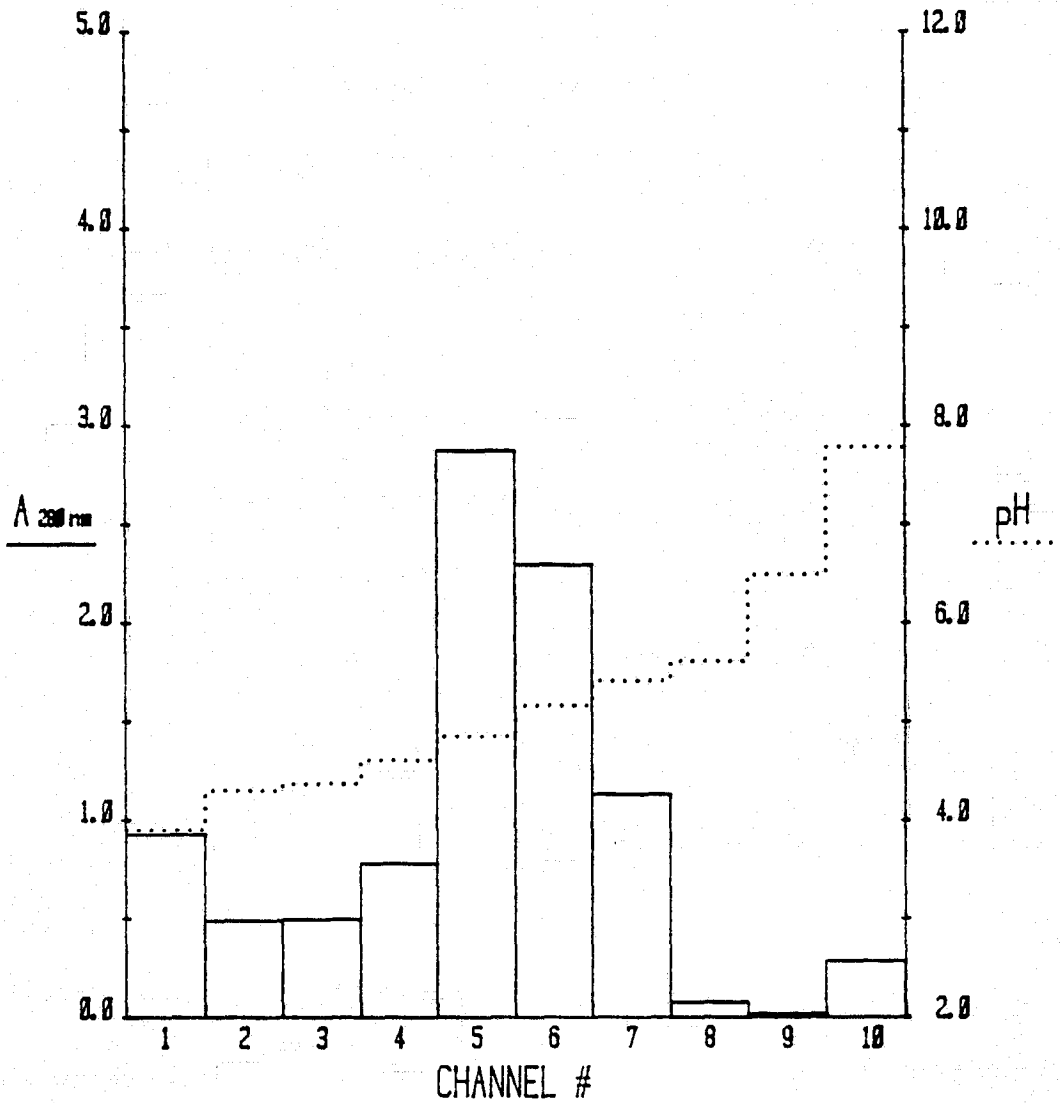


FIGURE 24

uv absorbance and pH vs channel of RIEF apparatus:
 results from fractionation of plasma containing a vasoactive
 peptide- the predominant component is serum albumin.

Laboratories for biological assay. The course of additional fractionation of these samples depends on the results of the biological assay for which we presently wait.

D. L-Asparaginase.

L-Asparaginase (from Abbott Laboratories), an enzyme that catalyzes deamination of L-Asparagine to aspartate and free ammonia, has been shown to be of medical value as an anti-lymphoma agent. Its action presumably results from the observation that susceptible lymphomas require asparagine. The protein is well characterized: M. W., 133,000 g/mole; isoelectric point, 5.2; structure, tetrameric; and it is stable unless highly purified, in which case, it is stabilized by high ionic strength. The assay method is based on measurement of release of ammonia from asparagine substrate.

The material, which contains at least 9 components within a 0.5 pH range (determined by PAG IEF) was first focused in a narrow pH range (pH 4-6) and then the three compartments containing significant proteins were subsequently resubmitted to RIEF. Ten fractions from the latter fractionation were analyzed for asparaginase activity and for protein distribution by PAG IEF. Results from these experiments are displayed in Figures 25-28. In this experiment Ampholine solution is prefocused without protein to establish the pH gradient. Figure 25 depicts the linear gradient formed in the focusing apparatus. L-asparaginase solution was then added to pH 6 (#7 reservoir) and focused for 2 hours. The protein distribution is shown in Figure 26. Since the majority of proteins focused into chambers 4-6, these three solutions were pooled and resubmitted to RIEF. The results of this run appear in Figure 27 where it can readily be seen that a linear,

UV ABSORBANCE and pH vs CHANNEL

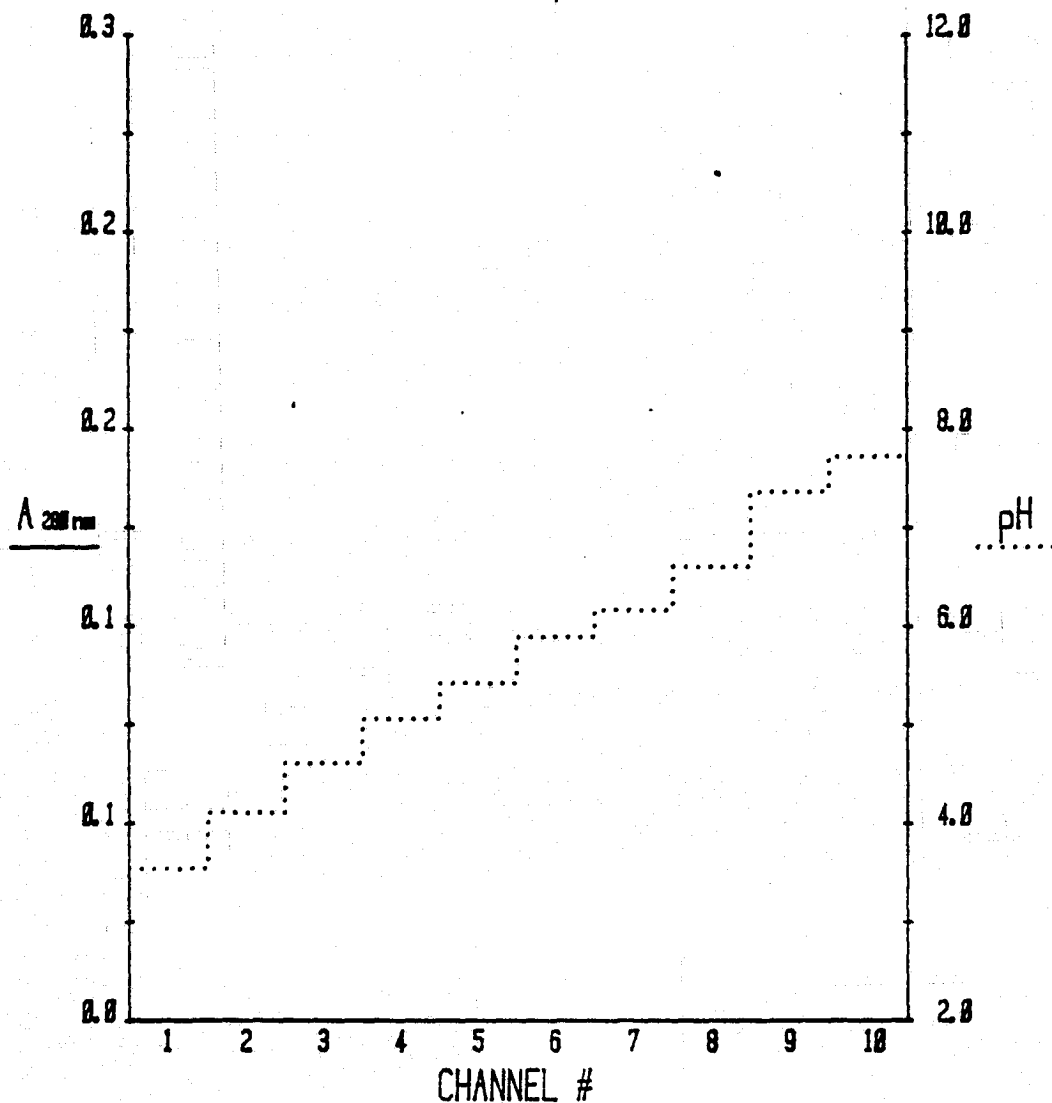


FIGURE 25

pH gradient established in the RIEF apparatus containing
0.08% Ampholine, pH 4-6, prior to addition of L-asparaginase

UV ABSORBANCE and pH vs CHANNEL

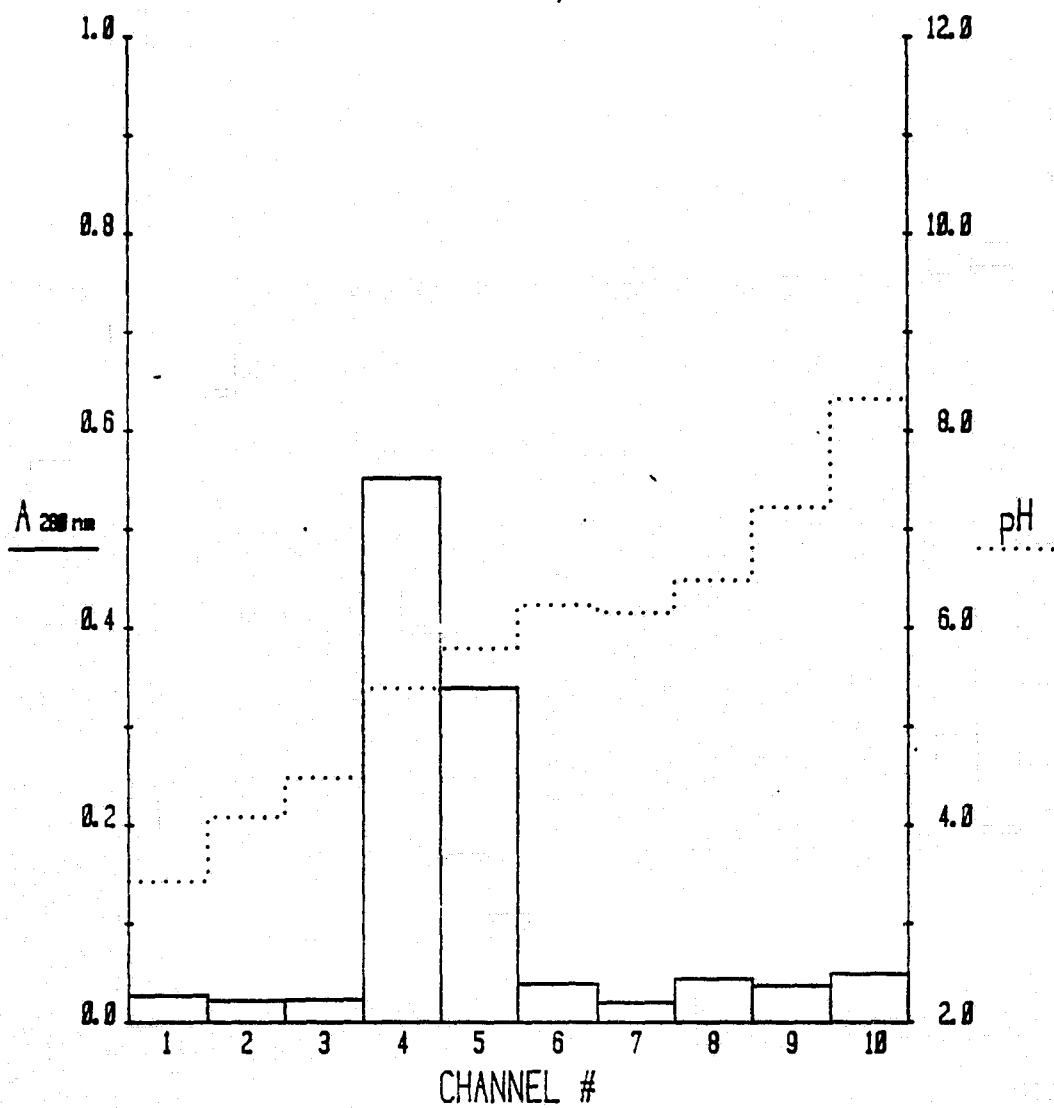


FIGURE 26

uv absorbance and pH vs channel following fractionation of L-asparaginase in a pH 4-6 gradient in the RIEF apparatus

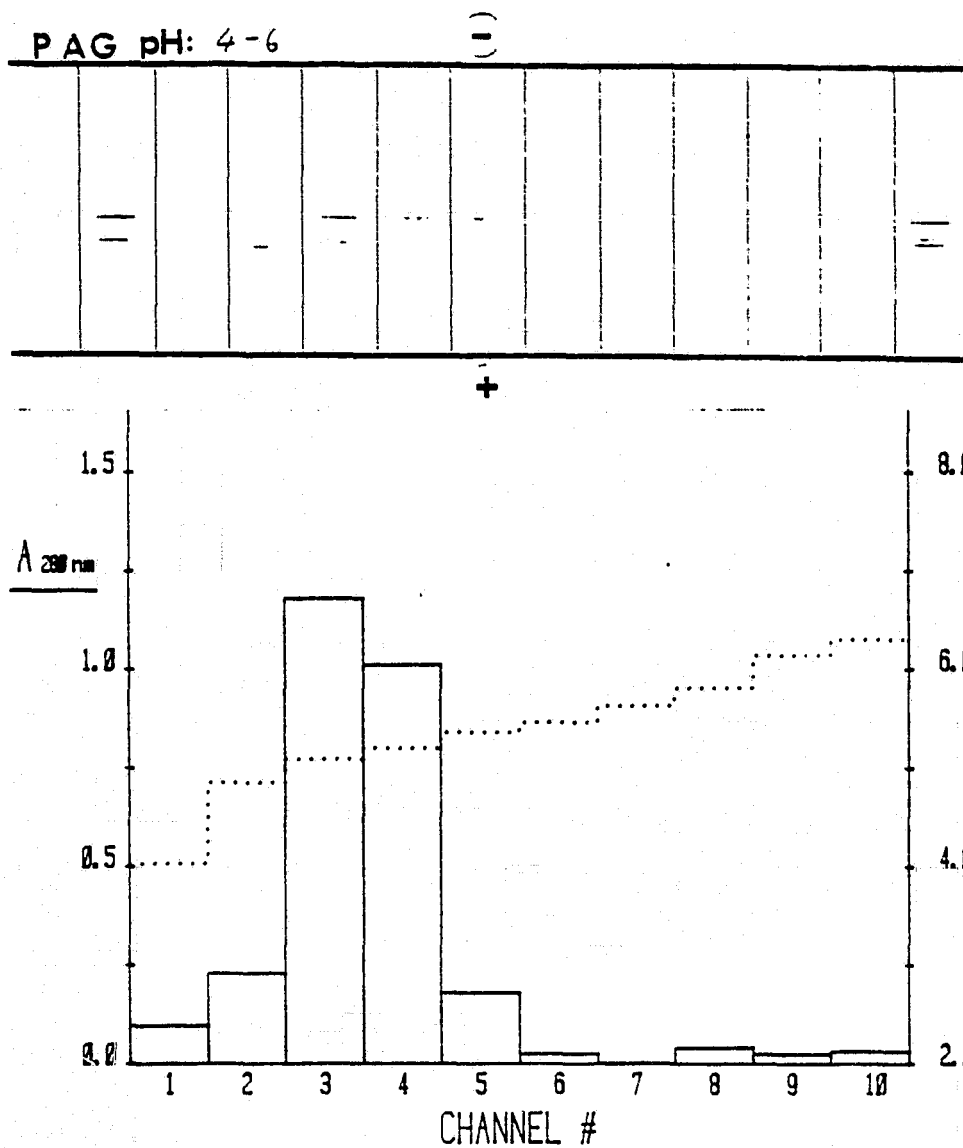


FIGURE 27 uv absorbance and pH vs channel following focusing of pooled fractions, pH 4-6. (from FIG. 26) in RIEF apparatus over narrow pH gradient (1 pH unit)

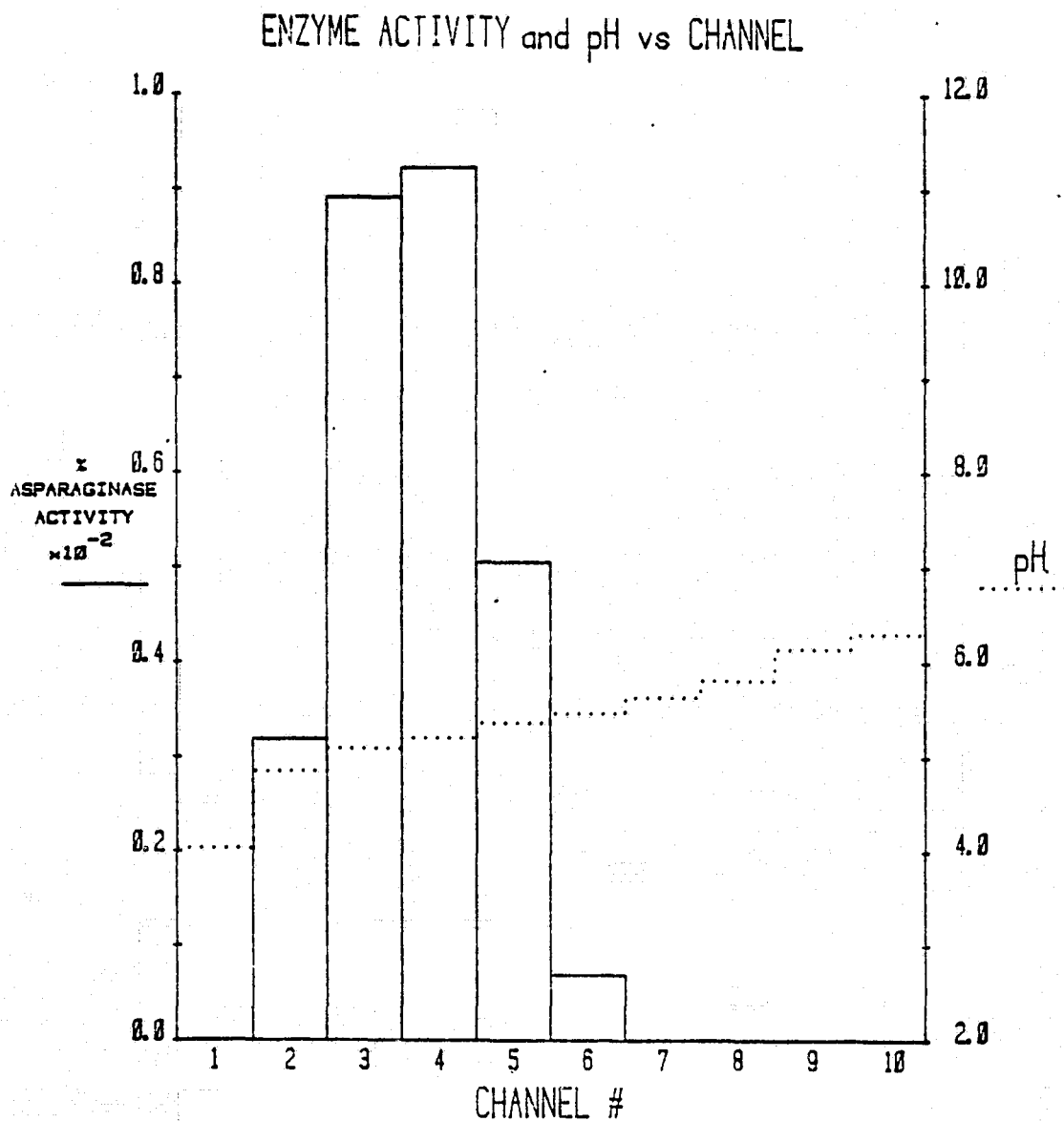


FIGURE 28 enzyme activity for RIEF fractions of L-asparaginase. Specific activity is reported as percent of starting material. Highest activity coincides with highest protein concentration (see FIG. 27)

one pH unit, gradient exists over the center 7 compartments, with significant protein focused in chambers 2-5. Appearing in Figure 27 is the protein distribution reproduced from PAG IEF analysis, which confirms the $A_{280\text{ nm}}$ measurements. L-asparaginase activity was measured (according to Worthington) as the release of ammonia from asparagine solution. Data from these measurements are reproduced in Figure 28 where activity per milligram of protein, calculated as percent of starting material, is shown for each fraction. The highest enzyme activity coincides with the highest protein concentration with no amplification of specific activity. This either results from activity being associated with a spectrum of proteins or a loss of activity upon isoelectric focusing.

E. ACP (Acid Phosphatase).

ACP, which catalyzes hydrolysis of phosphate esters is widely found in lysosomes of mononuclear phagocytic cells. Investigations of ACP is presently being conducted at the Laboratory of Cellular Immunology at the Tucson V. A. Medical Center by S. G. Axline and P. W. Cole. They are presently developing immunological assays for individual phosphatases and require milligram quantities of isolated fractions. The method of choice for preparative scale fractionation is granular, flat-bed IEF from which 100 μg batches can be cultivated per day. The goal of our collaboration is to extend the preparative scale 1000-fold, while maintaining a high resolution separation.

An initial attempt at purification of acid phosphatase by RIEF resulted in some success. ACP has a pI of 4.5-4.9, determined on PAG IEF. Twenty-five ml of mouse peritoneal macrophage lysate (cell lysis by Triton X-100, a non-ionic detergent) was dialyzed against three 800 ml changes of water over 36 hours at 4°C. The resulting

protein solution was applied to reservoirs #2-#4 of a prefocused pH 4-6 Ampoline gradient and focused at 200V for 1.5 hours. At this point there were negligible changes in current, pH, and $A_{280 \text{ nm}}$. Results from this focusing are illustrated in Figure 29. The absorbance in the anodal compartments is attributed to detergent. Enzymatic activity of ACP, determined by the α -NAP assay, resided in compartment 4 and accounted for approximately 25% of the total applied activity. An additional 25% of the applied activity was subsequently extracted from the paper filters. As noted in Figure 29, compartment 4 had a pH of 4.52 which is compatible with the reported pI for ACP. All fractions are presently being analyzed at the Tucson V. A. Medical Center by analytical IEF on flat-bed PAG to determine the resolution of protein purification. Preliminary results indicate that the RIEF system will provide rapid and reliable purification in preparative quantities.

It was concluded from the first separation that ACP requires detergent for solubility: ACP and the detergent migrated in opposite directions, resulting in the protein precipitate which collected on the filters. The detergent solution was introduced with the protein to reservoirs 2-4 equally. It is noted in Figure 29 that the detergent concentration (determined by $A_{280 \text{ nm}}$ with the protein concentration so low that it does not contribute to UV absorption) decreased with increasing distance from the anode. We interpret this detergent concentration gradient as electromigration of the detergent towards the anode. Experiments are planned in which steady state concentrations of detergent are maintained for protein solubility by constant infusion of detergent at the cathode.

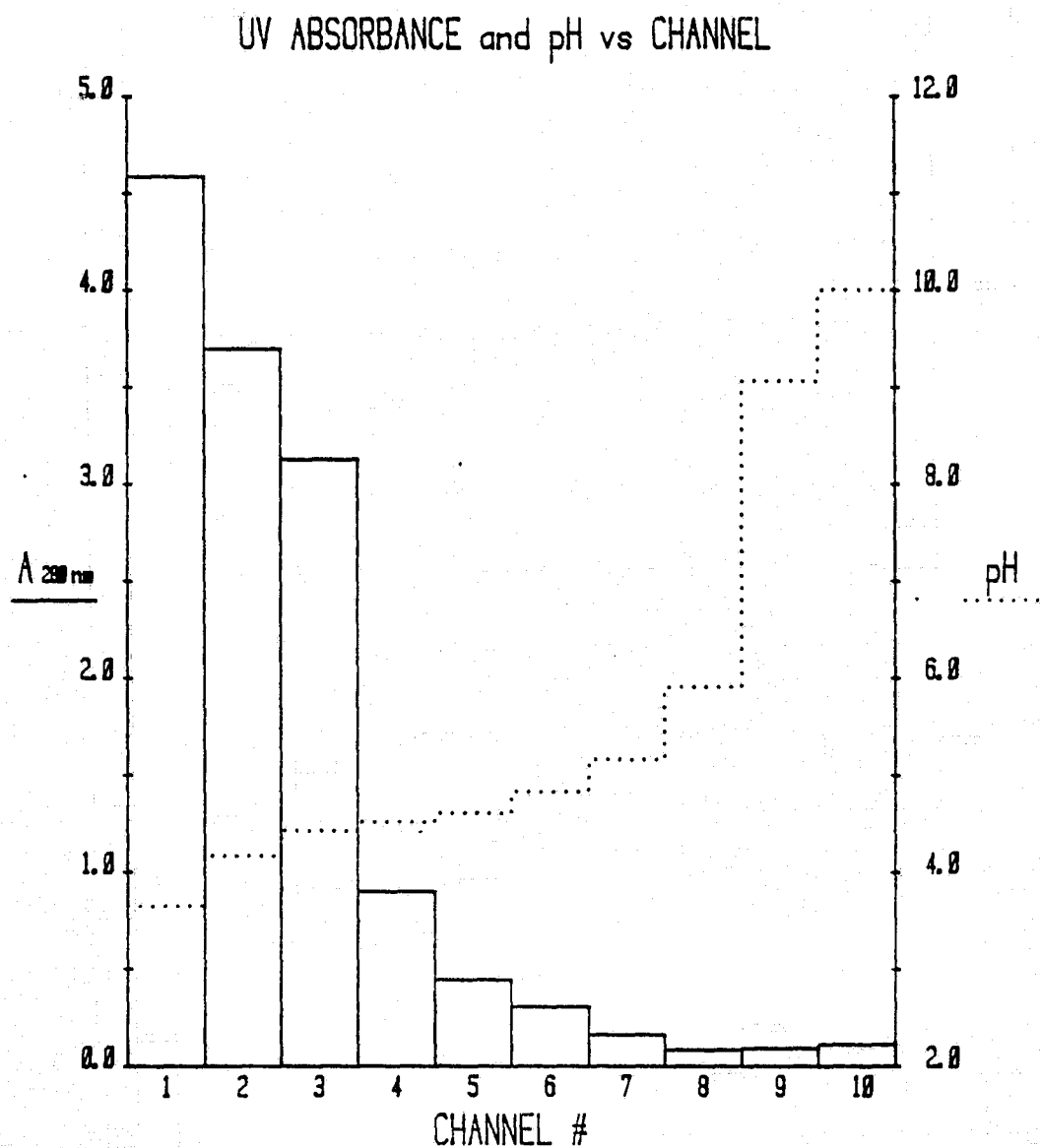


FIGURE 29 uv absorbance and pH vs channel: results from RIEF fractionation of ACP from mouse peritoneal macrophages. uv absorbance is attributed to detergent (Triton x-100)

IV. Detection of Peptides

One of our major objectives is to develop procedures for high resolution separation and purification of peptides and peptide hormones on a preparative scale by RIEF. An extensive list of possible candidates for this endeavor exists. Prior to submitting such peptides to preparative RIEF, analytical procedures for detecting peptides on PAG IEF must be developed since analysis of all fractions of the RIEF apparatus must be performed with this high resolution analytical technique. Some of the analytical procedures we are either presently employing or plan to develop include (1) staining, (2) fluorescent labeling of focused peptides, (3) fluorescent labeling of components prior to PAG IEF (see section IV. A. - Dansyl Derivatives of Amino Acids), (4) visible or ultraviolet densitometry (UV gel scanning), and (5) radiolabeling. Some of the peptides hormone candidates were focused on PAG and stained with Coomassie Blue in an attempt to determine the feasibility of these procedures in conjunction with preparative RIEF. The results of this work appear in Fig. 30 and demonstrate the applicability of RIEF with current analytical techniques for preparative focusing.

During this period, a great deal of activity was devoted to developing and adapting an existing analytical procedure for peptide detection on PAG and in solution. The analytical problem is associated with numerous difficulties based on the chemical similarities between peptides and carrier ampholytes: (1) Molecular weights of the two classes of compounds are frequently in the same range and separations based on this parameter become more difficult. Low molecular weights also render acid and basic denaturation impossible and peptide extraction rather

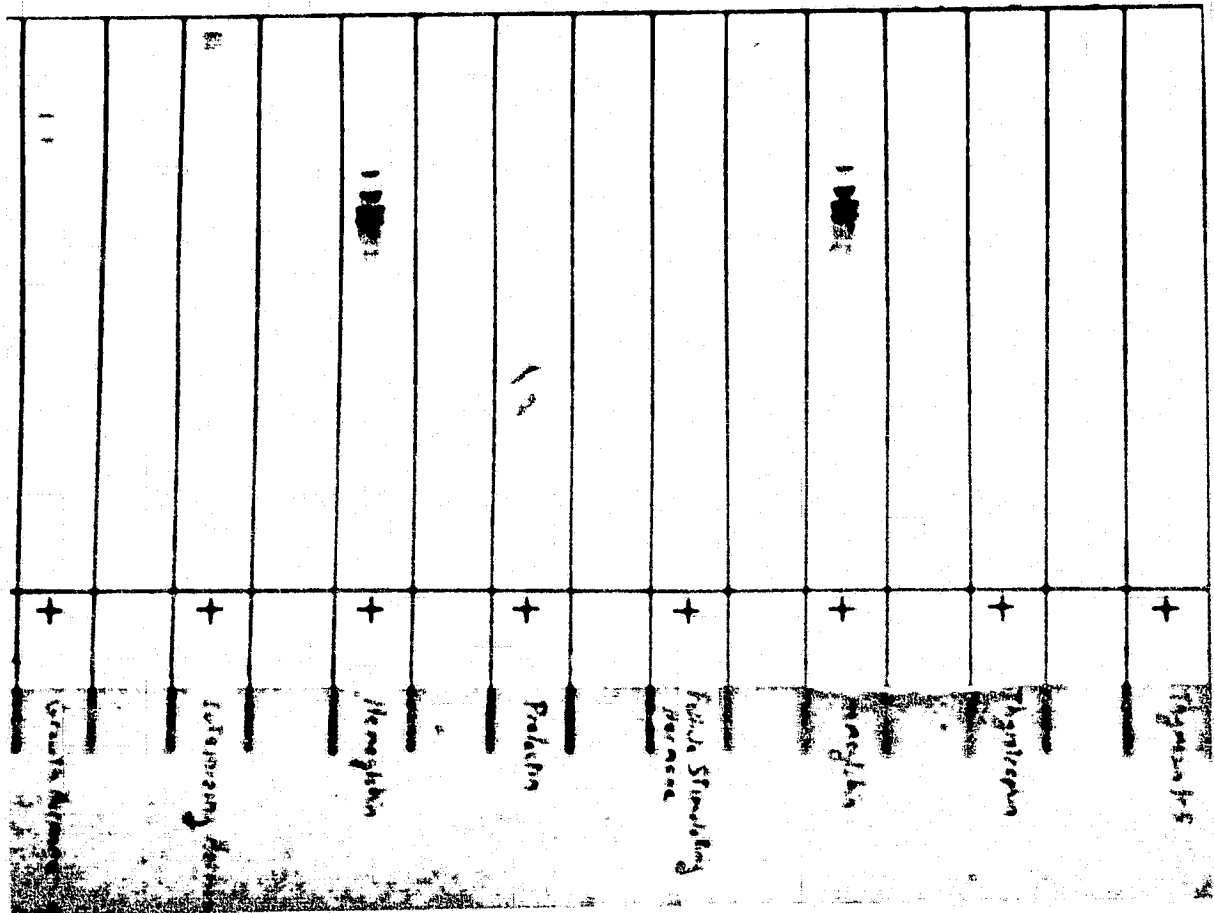


FIGURE 30

photograph of a PAG isoelectropherogram (pH 3.5-10) of
peptide hormones after staining with Coomassie blue

than precipitation occurs. This difficulty precludes fixation before staining on PAG. (2) Staining procedures in which colored stains couple to peptides through either basic or acidic functional groups also stain carrier ampholytes (containing identical functional groups) and sample-to-background stain ratios approach unity. (3) UV absorption of peptides depends, to a large extent, on the presence of Trp residues. With low MW peptides, Trp residues are frequently absent and spectrophotometric measurements based on UV absorption are impossible. Ampholines also absorb UV light to a slight extent and small amounts of weakly absorbing residues (Tyr) in peptides, render peptides indistinguishable from background absorption. (4) The aqueous solubility of ampholine and peptides are similar. Techniques such as solvent extraction to selectively remove the Ampholine from a focused peptide fraction is without high selectivity.

A. Dansyl Derivatives of Amino Acids and Peptides.

Dansyl chloride is commonly used in analytical biochemistry for detection of amino acids, peptides, and proteins. Its usefulness results from the fluorescent nature of the dansyl moiety of the derivatized compounds. Fluorescence enhances the limit of photometric detection by at least an order of magnitude resulting in an extremely sensitive quantitative analytical method. The qualitative nature of this reagent, at the present time, has been of less importance due to the relative insensitivity of the fluorescent emission wave length to the various dansyl derivatives. It is shown below that dansyl derivatives of amino acids and peptides offer a novel and sensitive method for qualitative as well as quantitative analysis using the IEF technique.

Amino acids and peptide derivatives of most fluorescent and color-ometric reagents are not suitable for IEF since the reagents couple to the amino groups with simultaneous loss of the ampholytic nature of the amino acid or peptide. This situation does not occur with dansyl since the dansyl group possesses a basic functional group (dimethylamine) and although the base of the amino acid is destroyed the resulting derivative possesses a base associated with the dansyl portion of the molecule. The isoelectric point for amino acids is generally midway between $pK-NH_2$ and $pK-COOH$ and therefore is not sharp (extending over 4 pH units). The pK of dimethylamine is significantly lower than that of the amino group and the pH difference between acidic and basic pK s of the dansyl derivative is smaller than in the original compound. This results in acidic derivatives with sharp isoelectric points.

Dansyl derivatives of 21 common amino acids were prepared and focused on a broad pH range PAG plate. Preliminary results from these runs were discouraging due to the lack of resolution between focused bands: all bands focused at the anode. Various dansylated amino acids were then focused on narrow pH range PAG plate (2.5-4, 3-5, 3.1-4.3, 2-6). Initial experiments using narrow range acidic gels were encouraging since separation between dansylated amino acid was observed. However, diffusion rates of focused bands were high (band lengths increased with increasing focusing time) and anodic drift of focused bands was observed. Experimental techniques to minimize diffusion were investigated. Thus 8M urea was incorporated in the gels; however, reduced fluorescence was observed. To minimize anodic drift, various Ampholine solutions were used as electrode electrolytes; however, decreased resolution of closely spaced bands resulted with all but strong acid and base as anolytes and catholytes.

An additional barrier to high resolution focusing of dansyl amino acids is caused by weak buffer capacity of commercial carrier ampholytes at the extremes of pH. The manufacturer of Ampholine suggests the use of 1% (w/v) concentration in PAG. We found it necessary to increase Ampholine concentration in order to improve resolution.

The conditions for successful separations of dansyl amino acids were (1) narrow pH range acidic gels (pH 2.5-4, 3-5, or 3.1-4.3), (2) 2.5-3% (w/v) Ampholine concentrations, (3) 10 mg amino acid/focused band, (4) strong acid and base electrode solutions, and (5) short focusing times. Figure 31 is a photograph of a focused PAG plate, pH 3.5-5, illuminated with long wave length ultraviolet light. Dansyl derivatives of 21 amino acids appear as fluorescent bands along the diagonal axis. Acidic and basic byproducts (e.g. hydrolyzed dansyl chloride) of each reaction are also present in each lane. A schematic representation of this same plate is presented in Figure 32. Only the dansyl amino acid bands have been included. Superimposed on this figure is the pH profile from pH measurements of aqueous extracts of gel slices. It can be seen that the pH range of the focused bands is 3.2-3.8 with 15 mm gel length between the extreme components. An identical separation was recorded on a pH 3.1-4.3 PAG plate (in which the distance between extremes was almost doubled). Resolution between focused adjacent bands was also improved with the narrow pH range.

It can be seen from Figure 31 that the dansyl derivatives of amino acids were arranged on the gel in order of increasing pI. In order to determine whether a distinction between compounds with closely spaced pI's could be discerned, binary mixtures of dansyl amino acids

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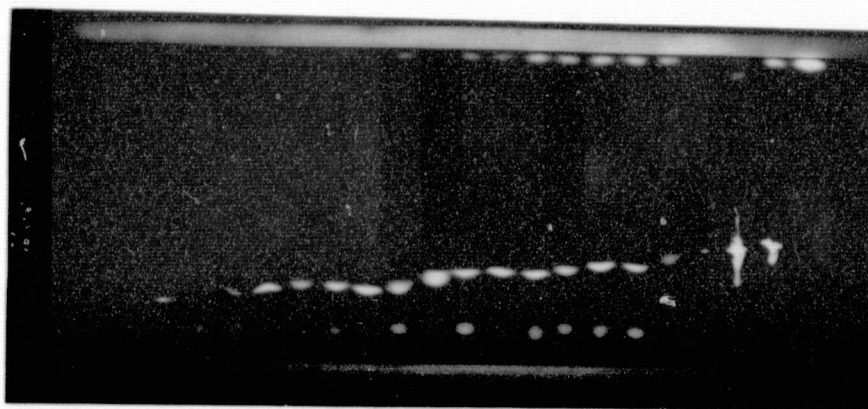
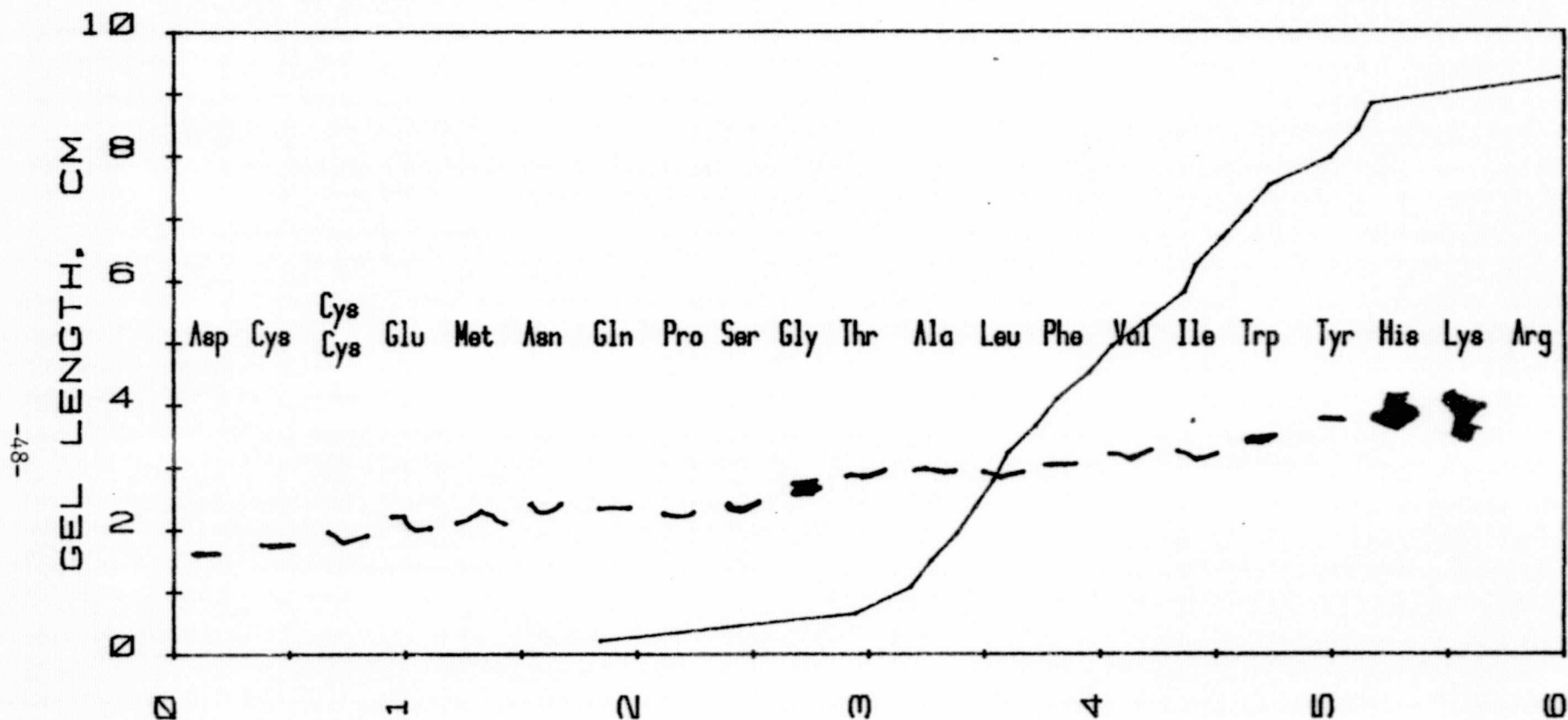


FIGURE 31

photograph of focused PAG plate, illuminated with uv light, containing dansyl derivatives of 21 amino acids. Dansyl amino acids appear as fluorescent bands along the diagonal axis.



pH
FIGURE 32

schematic representation of PAG isoelectropherogram, reproduced from FIG. 31.
pH profile of gel is superimposed to indicate pI of focused bands.

were prepared. These solutions contained dansyl amino acids of nearest neighbors and next nearest neighbors. The table below illustrates the results of the focused mixture. (Nearest neighbors refers to a compound and the one appearing immediately below it in the table. Next nearest neighbors refers to a compound and that appearing two lines below). A + sign indicates two bands can be discerned: -, only one band is observed for the mixture.

<u>Separation of next nearest neighbors</u>	<u>Dansyl Amino Acids (increasing pI)</u>	<u>Separation of nearest neighbors</u>
-	Asp	-
+	Cys	-
-	Cys-Cys	-
+	Glu	-
-	Met	-
+	Asn	+
-	Gly	+
+	Pro	-
+	Ser	+
+	Gly	-
-	Thr	-
-	Ala	-
+	Leu	+
+	Phe	+
+	Val	-
-	Ile	+
-	Trp	-
-	Tyr	-
-	His	-
	Lys	-
	Arg	

One additional PAG plate (pH 2.5-5-4) containing various 2 component mixtures of dansyl amino acids was focused. The mixtures were selected for the chemical similarities of the component parts. The twenty mixtures

are presented in the table below and the photograph of the focused plate, illuminated with UV light, appears in Figure 33. Included in this table is the notation (+ or -) indicating whether the mixture is separable into component parts by this technique.

<u>Mixture of Dansyl Amino Acids</u>	<u>Separable on PAG IEF</u>
Asp+Glu	+
Asp + Asn	+
Cys + Ala	+
Cys + Met	+
Cys-Cys + Asn	+
Glu + Gln	-
Met + Pro	-
Gln + Gly	+
Pro + Hyp	+
Ser + Thr	+
Gly + Ala	+
Thr + Phe	+
Ala + Val	+
Leu + Val	+
Leu + Ile	+
Phe + Trp	+
Phe + Tyr	-
Ile + His	-
Trp + Lys	-
Tyr + Arg	-



FIGURE 33

photograph of focused PAG plate, illuminated with uv light,
containing dansyl amino acid pairs (see preceding table),
selected for chemical similarity of its components

An attempt at further increasing the distance between adjacent focused bands by placing the electrodes so that focusing occurs in the long gel length (29 cm) was unsuccessful. By doubling the distance of migration the time required for focusing was also increased and diffusion of focused bands in the direction perpendicular to the axis of electromigration significantly increased. The result was wide bands of decreased concentration and low fluorescence.

B. Fluorescent Labeling of Focused Amino Acids, Peptides and Proteins.

A major consideration of our work was developing an analytical system for detecting low M.W. peptides after isoelectric focusing on PAG. Fluorescent staining techniques employed before focusing generally modify the pI and therefore change and complicate the outcome of an experiment. It is for this reason our initial attempts to visualize peptides were undertaken with focused gels. After a lengthy period of unsuccessful attempts of developing focused peptides, we redirected our efforts towards prelabeling the compounds with dansyl groups (described in the preceding section).

A brief description of unsuccessful attempts of visualizing focused proteins and peptides with a visual stain (Coomassie Blue) and fluorescent labels (Fluram and Fluoropa) follows:

1. Submersion of focused gel plates in aqueous Fluoropa-
A focused PAG plate (pH 3.5-10) with 20 μ g each of (amino acids) Lys, Gly, Pro, Tyr, Glu, Met, Cys-Cys, Arg, Phe; (peptides) glutathione, Ala-Ala, His-His, Oxytocin, Thymosin; and (proteins) Hb, Alb, ED, and AHF was submerged in 3.7 mM Fluoropa at pH 10.4 (borate). No fluorescent bands were discerned within 24 hours; however, Ampholine in the gel immediately reacts with the reagent and a bright

fluorescent background resulted. In a control study it was demonstrated that each compound listed above quickly reacts in Fluram solution yielding fluorescent derivatives. We conclude that fluorescent bands were not visualized due to masking by the bright fluorescent background, rapid extraction of focused bands, or components within the gel that interfere with the Fluram-amino reaction.

a. One variation of the above procedure was attempted to increase contact of focused bands with Fluoropa solution. Narrow slices of focused gels (parallel to the direction of electromigration) were submerged in Fluram solution. Fluorescent bands of focused compounds could not be discerned from the bright fluorescent background.

b. Another variation of the same theme was performed by innoculating the focused gel with Fluram solution. The attempts were unsuccessful.

c. A related experiment was performed to determine whether the Fluram-amine reaction could be performed at a pH which would permit Fluram-peptide or Fluram-amino acid reaction to occur at faster rates than the Fluram-Ampholine reaction and thus eliminate the bright fluorescent background. All the above compounds as well as Ampholine solution were reacted with Fluoropa solution at 0.5 pH intervals between pH 7-11. No observable difference between the rate of formation or the intensity of fluorescence could be distinguished between Ampholine and other amino-containing compounds.

2. Diffusion of focused bands - A focused PAG plate identical to that in section 1 above was held in close contact with Fluoropa soaked filter paper in an attempt to develop fluorescence

of focused bands at the gel/paper interface by diffusion of focused bands. No distinct bands were developed and again a bright background was observed.

A variation of the above procedure was performed by maintaining Fluoropa soaked filter paper in close contact with the focused PAG plate while maintaining a focusing current with 100V. This was performed to limit diffusion of protein to the gel-paper axis.

3. Freeze drying/solvent extraction/staining of focused gels - PAG plates containing various proteins and peptides were freeze dried to fix focused bands. (We experimented with gels of different thicknesses to optimize the ease of freeze drying and solvent extraction. Gels of 0.03 inch appear to give better results than thicker gels supplied by manufacturers of PAG equipment). Various solvents were employed in an attempt to selectively extract Ampholine from focused proteins and peptides from the freeze dried gels. Some of the solvents employed include:

- ethanol - extracts proteins, peptides and Ampholine
- acetone - does not extract Ampholine
- acetone : ethanol 1:1 - does not extract Ampholine
- acetone : ethanol 1:3 - does not extract Ampholine
- cyclohexane - does not extract Ampholine
- acetylacetone - does not extract Ampholine
- methylethyl ketone - does not extract Ampholine

C. Ampholine Extraction from Focused Gels.

1. TCA/acetone (Fixation/Extraction) - Focused PAG plates were submerged in 30% TCA solution in acetone in an attempt to fix protein while simultaneously extracting Ampholine. In a typical experiment even large proteins, e.g. Hb, Alb were extracted from the

gel. This procedure, however, apparently requires careful determination of TCA concentration tailored to individual proteins. This investigation was discontinued since it would not be sufficiently general for a routine analytical procedure.

2. Dehydration/Ampholine removal by solvent extraction - PAG plates with focused proteins were submerged in solvents (e.g. ethanol, acetone) to remove water and/or Ampholine from gels. In general the results were negative since most proteins were extracted into solvents. When proteins were fixed, Ampholine was not completely extracted. An exception was noted only for the protein albumin which fluoresced on Fluoropa rehydration of a solvent - extracted and dehydrated gel.